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Notch receptors play important roles in both normal animal development and pathogenesis. We show that the CDC4 family protein SEL-10 negatively regulates Notch receptor activity by targeting the intracellular domain of Notch receptors for ubiquitin-mediated protein degradation. The WD40 repeats of SEL-10 interact with the C-terminal domain of Notch receptors. SEL-10 binds preferentially to a phosphorylated form of Notch4 in cells, and mediates ubiquitination of Notch proteins in vitro. Blocking endogenous SEL-10 activity by over-expression of a dominant-negative form of SEL-10 with only the WD40 repeats leads to stabilization of the intracellular domain of Notch and up-regulation of Notch induced reporter activation.

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Notch4 Ligands in Mammary Gland Development and Tumorigenesis

Table of Contents

Introduction

I. Nature of the Problem

Notch/lin-12 receptors have been shown in many cases to regulate cell fate decisions. Abnormal activation of some of these receptors is also linked to tumorigenesis. Among all the Notch/lin-12 family members, mouse Notch4/int-3 is the only one that has been reported to be expressed in mammary epithelium, and to regulate mammary gland development and tumorigenesis.

A few ligands of the Notch/lin-12 receptors have been identified and as expected, have been shown to regulate cell fate decisions. However, none of these ligands have been investigated for their role in tumorigenesis or mammary gland development. As for Notch4, the only Notch/lin-12 receptor associated with mammary gland development and tumorigenesis, no ligand has been identified yet.

My proposed study intend to identify Notch4 ligands, study the nature of their interactions with Notch4 receptor and determine their roles in mammary gland development and tumorigenesis. The results of my research will shed light on Notch signaling and mammary gland biology from a new angle.

In addition to the studies on Notch receptors ligands, my collaborators and I identified a novel gene, *sel-10*, as a negative regulator of *Notch/lin-12* activity. Due to the importance of *int-3* as an oncogene involved in mammary tumorigenesis and the potential of *sel-10* as a therapeutic tool against *int-3*, I carried out experiments to understand the molecular mechanisms by which SEL-10 regulates Notch. My

results demonstrate that SEL-10 inhibits Notch by targeting Notch receptor for ubiquitin-mediated protein degradation. This part of my research, although not originally proposed in my Fellowship Application, has provided the same kind of scientific training required by the Fellowship. The results of this research are presented in this report under Specific Aims 3, 4, and 5, which are new additions to the two original Specific Aims.

II. Background

Notch4/int-3 proto-oncogene was cloned by our lab. *Notch4* gene encodes a 220KD protein product, which bears all the hallmarks of a *Notch/lin-12* gene family member. This protein product is expressed in mouse embryos and a number of adult tissues including lung, kidney, heart and mammary gland(1, 2). *In situ* hybridization data showed that Notch4 expression in day 13.5 embryo and adult lung is endothelial cell specific(1). Our immunofluorescent staining data (unpublished) have confirmed that Notch4 is a cell surface localized protein.

Truncated Notch proteins have been shown to be involved in tumorigenesis. In *Drosophila* and other organisms, truncated Notch proteins containing the intracellular domain behave like constitutively activated receptors(3). The truncated form of *TAN-1* gene was first isolated from patients with acute T lymphoblastic leukemia(4), and was later shown to promote T cell neoplasm in bone marrow reconstitution assay(5) and to transform rat kidney cells in soft-agar assay(6). The truncated *Notch4* gene, *int-3* was identified as a target of integration by mouse mammary tumor virus (MMTV) in mouse mammary tumors. Viral integration into the *int-3* gene results in the expression of a truncated 2.4kb transcript(7). Transgenic mice that express *int-3* under the control of either MMTV LTR or whey acidic protein (WAP) promoter develop poorly differentiated mammary

adenocarcinomas at 100% penetrance(8, 9). Histological examination showed that the mammary glands of the transgenic mice were arrested during development and were lactation deficient(8). int-3 has also been demonstrated to be oncogenic in cultured mammary epithelial cells(7).

Although Notch receptors activated by truncation have been extensively studied for their role in development and tumorigenesis, Notch receptors activated by ligand have never been analyzed in the same scenario. Genetic and molecular analyses have identified several Notch ligands in *Drosophila* and *C. elegans*. *Drosophila Delta* and *Serrate* genes and *C. elegans Lag-2* and *Apx-1* genes encode a family of structurally related ligands for the *Drosophila* Notch and the *C. elegans* lin-12 and glp-1 receptors(10, 11). These ligands are all membrane-bound proteins of which the extracellular domains contain a variable number of EGF-like repeats and a cysteine-rich DSL (Delta-Serrate-Lag-2) motif. Although in general these structural motifs are believed to participate in ligand-receptor interaction, it is not very clear what specific regions are involved in receptor binding and how they interact with the receptor. The function of the very short intracellular domain of Notch ligands is not clear either. Notch ligands have also been identified in mammals. *Jagged-1*, a rat homologue of *Serrate*, was cloned from Schwann cell cDNA library by low stringency hybridization(12). It has all the typical structures of Notch ligands. Jagged-1 is co-expressed with Notch1 in developing spinal cord and other tissues. Jagged-1 activated Notch1 blocks in vitro myogenesis in a way very similar to the truncated receptor. Other mammalian Notch ligands, such as human Jagged-1 and Jagged-2, mouse Delta-like 1 (Dll1) and Dll3 have also been cloned and shown to be involved in different developmental processes. For example, human Jagged-1 mutations have been found to be responsible for Alagille syndrome, a genetic disorder characterized by abnormal development of liver, heart, skeleton, eye, face and kidney. However, the role of these mammalian Notch receptors in tumorigenesis has never been determined. *C. elegans* Apx-1, although has different

receptor and different mutant phenotype from Lag-2, can fully substitute Lag-2 when expressed under the control of *lag-2* regulatory sequences(13). Similarly, *Drosophila* Serrate can functionally replace Delta during neuroblast segregation in the *Drosophila* embryo(14). This implicates that different Notch receptors and ligands function through a similar mechanism.

Genetic and molecular studies have also identified intracellular components of Notch signaling pathway. *Drosophila* gene *Suppresser of Hairless (Su(H))* may play a central role in Notch signaling. Su(H) protein is sequestered in the cytoplasm when co-expressed with Notch protein in cultured *Drosophila* S2 cells and is translocated to the nucleus when Notch binds to its ligand Delta(15). In mammalian cells, truncated mouse Notch1 and Notch2 have been shown to be localized in the nucleus and interacts directly with RBP-Jκ (recombination signal sequence binding protein for Jκ genes), a transcriptional factor highly related to Su(H). The binding of truncated Notch1 and Notch2 to RBP-Jκ activates the expression of RBP-Jκ repressed genes, such as Hairy Enhancer of Split (HES-1) (16, 17, 18). The fact that truncated Notch proteins without the extracellular domains are always localized to the nucleus has led to the speculation that a cleaved fragment of wild-type Notch receptor may participates directly in the downstream nuclear events of Notch signaling. A few studies have provided very strong evidence to support this model(19, 20).

Besides Su(H) and RBP-Jκ, not much is known about the intracellular regulation of Notch signaling. In collaboration with Dr. Iva Greenwald's lab, we identified a *C. elegans* gene *sel-10* as an intracellular negative regulator of *lin-12* activity(21). Sequence analysis indicates that SEL-10 contains one F-box domain and seven WD40 repeats, typical features of a CDC4 family protein, which are known to promote ubiquitin-mediated protein degradation. Previous studies on CDC4 demonstrate that CDC4 is a component of the

ubiquitin-ligase complex, and its main function is to determine target specificity by binding to phosphorylated target proteins. CDC4 binding triggers the formation of covalent bond between ubiquitin and the target protein. Once ubiquitinated, the target protein can be rapidly degraded by 26S proteasome. Since, co-immunoprecipitation assays showed that C. elegans SEL-10 complexes with LIN-12 and with murine Notch4, we proposed that SEL-10 promotes the degradation of LIN-12/Notch proteins by the ubiquitin-proteasome pathway.

III. Purpose

The original goal of my proposed work is to identify Notch4 ligands and determine their role in mammary gland development and tumorigenesis.

The additional goal of my research is to understand the molecular mechanism underlying the regulation of Notch/LIN-12 by SEL-10.

Body

I. Technical Objectives

The first goal of my research is to identify ligands for mouse Notch4 receptor, understand the molecular details of their interactions with Notch4, and determine their involvement in mammary gland development and tumorigenesis. I proposed specific aims 1 and 2 to achieve this goal in a period of three years.

The second goal of my research is to study the functional and physical interactions between SEL-10 and Notch/LIN-12 receptors at the molecular level, and to provide evidence supporting our theory that SEL-10 promotes Notch/LIN-12 ubiquitination. I proposed specific aims 3, 4 and 5 for this goal.

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

In mouse, several genes have been identified as Notch ligands, such as *Jagged* and *Delta-like* genes. Since some Notch ligands are interchangeable, we will test whether the protein products of these genes are able to interact with and activate Notch4 receptor.

In addition, we will try to identify novel putative ligands for Notch4 by a screening strategy. Extracellular domain of Notch4 receptor will be used as a molecular probe to screen an eukaryotic expression library from mouse mammary gland. Once a novel gene is identified, we will obtain its full length cDNA for further investigation.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

To demonstrate that a candidate protein is the bona fide Notch4 ligand, we will first show its physical interaction with Notch4 receptor by molecular and cellular techniques. As a property of EGF repeat contain proteins, their interaction should be Ca^{++} dependent. We then plan to analyze the expression patterns of the candidate ligands and Notch4 receptor. This experiment will not only help us to rule out ligands for other Notch receptors that can cross-interact with Notch4 but also inform us whether Notch4 has different ligands in different tissues. Furthermore, by functional assays, we will test the candidate ligands for their ability to activate Notch4 signaling pathway. Finally, we will also test Notch4 ligands for their ability to regulate mammary epithelial cell development by using transformation and differentiation assays.

Specific Aim 3: Mapping the Domains Required for SEL-10/Notch Interaction.

I plan to use a series of deletion mutants of mouse Notch4 and human SEL-10 in co-immunoprecipitation assays to address which domains are involved in the binding between the two proteins. I will also test if Notch4 can be phosphorylated and if phosphorylation is required for Notch binding to SEL-1. I will learn from the results of these experiments whether the molecular nature of Notch/SEL-10 interaction is consistent with what has been established by the studies of CDC4.

Specific Aim 4: Ubiquitination of Notch Proteins.

To answer whether SEL-10 can promote Notch ubiquitination, I will first address if Notch can be ubiquitinated in the cell. One approach I will use is to test if the stability of

Notch proteins can be affected by specific inhibitors of proteasome. Another approach is to precipitate 6XHis tagged Notch proteins from the cell under denaturing conditions and probe for covalently linked ubiquitin with a ubiquitin specific antibody.

Specific Aim 5: Regulations of Notch Ubiquitination and Function by SEL-10.

Once I establish that Notch can be ubiquitinated in the cell, I will conduct in vitro ubiquitination assays to study if Notch ubiquitination can be mediated by SEL-10. I will also carry out a luciferase reporter assay to see if Notch activity can be regulated by SEL-10. To test if the WD40 repeats of SEL-10 can stabilize int-3 by blocking full length SEL-10 function, I will carry out pulse-chase experiment to study if the half-life of int-3 can be prolonged and if the steady state levels of int-3 can be increased by the WD40 repeats.

II. Experimental Results

This report describes the progress I have made during the 36 months of this fellowship. The progress reported here will be related to the original tasks set out in the Statement of Work, and the newly added specific aims 3, 4 and 5.

Specific Aim 1: Identifying and Cloning Genes that Encode Putative Notch4 Ligands (MONTHS 1-18)

A. Obtaining full length cDNA clones of mouse Jagged and Delta-like 1. We have obtained rat Jagged-1 cDNA, epitope-tagged it and cloned the tagged gene into retroviral vector and adenoviral vector. At the same time, we also cloned Notch4 and int-3 genes into retroviral and adenoviral vectors. These vectors have been successfully used to drive stable

or transient gene expression in different cells. A Western blot showing the expression levels of the three proteins is attached in Appendix A.

We have also acquired mouse Developmental Endothelial Locus-1 (Del1) gene which encodes a EGF repeat containing protein in endothelial cells, the same location where Notch4 is expressed. We will test Del1 as a candidate Notch4 receptor. We are also in the process of acquiring more candidate genes, such as mouse Delta-like 1 (Dll1) and Dll3, Jagged-2, and so on. All these candidate Notch4 ligands will be tested for their ability to interact with Notch4 bio-chemically and biologically.

B. Screening mammary gland eukaryotic expression libraries for putative Notch4 ligand. For this approach, we planned to determine the locations where Notch4 ligands are most concentrated and then screen an expression library made from such locations. Northern blot and in situ hybridizations have shown that Notch4 is expressed in endothelial cells. To confirm this result, we have been trying to study the expression pattern of Notch4 in adult mouse tissues by immunohistochemistry. A well established expression pattern of Notch4 will be a good indication of where its ligands are located.

We used a rabbit polyclonal antiserum against the C-terminal region of Notch4 to probe for Notch4 expression in adult mouse kidney tissue sections. The characteristic glomeruli with endothelial cell clusters in the renal cortex are very easy to identify, and that makes kidney a perfect organ to establish the conditions of immunohistochemical analysis using our antiserum. Together with anti-Notch4 antibody, we also used pre-immune serum in our immunohistochemical staining as negative control and anti-PECAM (an endothelial marker) antibody as positive control. Our preliminary results strongly indicate that Notch4 is expressed in kidney glomeruli. However, high background has prevented us from producing publication quality photos. To solve this problem, we are now trying to purify

our anti-Notch4 antiserum using affinity chromatography and making antibodies against other regions of Notch4.

The Notch1 ligand, Jagged-1 has been reported to be expressed in endothelial cells. It is natural to address whether Jagged-1 can also serve as a Notch4 ligand. In our immunohistochemistry studies using kidney sections, we also include an affinity purified Jagged-1 antibody. The staining pattern of Jagged-1 is clearly endothelial and is very similar to Notch4 expression pattern.

Specific Aim 2: Biochemical and Biological Interactions between Putative Ligands and Notch4 Receptor (MONTHS 18-36)

A. Co-immunoprecipitation of Notch4 and its putative ligands. We are in the process of testing Notch4 and Jagged-1 in co-immunoprecipitation experiments. Jagged-1 has been HA-tagged and will be co-expressed with Notch4 by transient transfection into Bosc23 cells. Anti-Notch4 and anti-HA antibodies will be used to precipitate and detect the protein products.

B. Cell aggregation assay to show physical interactions between Notch4 receptor and ligands. This experiment has not been carried out yet. But we have gathered all the necessary reagents, and once a promising candidate gene is available, we will test it in this assay.

C. Transformation assays to show activation of Notch4 receptor by its ligands.

D. Differentiation assays to show activation of Notch4 receptor by its ligands.

We have not carried out experiments proposed in C and D. Instead, we have developed a luciferase assay to test Notch4 candidate ligands in a faster and more quantitative way. Once a Notch4 ligand is identified in the luciferase assay, we eventually will test its role in mammary gland development and tumorigenesis by transformation and differentiation assays using mammary epithelial cells. In our preliminary studies, we were unable to show conclusively whether Jagged-1 can activate Notch4 and lead to luciferase expression. We are in the process of modifying the experiment to get more consistent results.

E. Immunofluorescent staining to study the subcellular localization of Notch4 receptor before and after its activation by ligand. Recently studies have provide quite convincing evidence supporting the model that Notch activation by its ligands induces a proteolytic processing resulting in the translocation of the intracellular domain of the receptor to the nucleus. Only a small amount of the truncated protein is required to exert its nuclear function. That is why it is very hard to detect the nuclear fragment caused by ligand binding. We still plan to do the proposed immunofluorescent staining experiment at a convenient time. But it will not be a top priority in the overall plan.

F. Luciferase assays to show activation of Notch4 receptor by its ligands. This experiment was not proposed in my original proposal. The intracellular domain of Notch1 has been shown to be able to activate a luciferase reporter gene under the control of a HES-1 promoter(22). We replaced Notch1 intracellular domain with int-3 or Notch4 and showed that int-3 can activate HES-1 transcription while Notch4 can not. We are going to co-express Jagged-1 and Notch4 in HELA cells using adenovirus and determine if Jagged-1 can activate Notch4 and lead to luciferase expression. This experiment is much faster and easier to carry out than transformation and differentiation assays. It will be our major approach to identify Notch4 ligands.

G. Cross-regulation among Notch receptors and their ligands. This experiment was not proposed in my original proposal. In our experiments designed to study the function of Notch receptors in endothelial cells, we found that the expression of one Notch receptor or ligand can sometimes up-regulate or down-regulate the expression levels of other Notch receptors or ligands. For example, we have found by Northern blot that Notch1, Notch3, Notch4 and Jagged-1 are all expressed in RBE4 cells, a rat brain endothelial cell line. Exogenous expression of the activated form of Notch4, int-3 increases the steady state levels of both endogenous Notch4 and endogenous Jagged-1 (Appendix B). We will assess this cross-regulation in further details and try to determine the specificity among Notch ligands and receptors. This study has the potential to develop into a new functional analysis system for Notch ligand-receptor interaction.

Specific Aim 3: Mapping the Domains Required for SEL-10/Notch Interaction

A. Interaction between int-3 and human SEL-10. I have shown through co-immunoprecipitation assays that int-3 can not only bind to C. elegans SEL-10 (Appendix C, figure 4), it can also form a complex with human SEL-10 (Appendix C, figure 4). The experiment was done by transfection of Bosc23 cells with HA-tagged int-3 and myc-tagged human SEL-10, followed by immunoprecipiation and Western blotting using the corresponding antibodies.

B. Domains of int-3 and human SEL-10 required for their interaction. I generated a series of deletion mutants of int-3 and human SEL-10 and tested them in co-IP assays to investigate which domains are responsible for the binding between int-3 and human SEL-10. My results indicate the C-terminal region of int-3 after the CDC10/ankyrin repeats and

the WD40 repeat region of human SEL-10 are the domains involved in their interaction (Appendix D, figures 2A and 3).

C. Interaction between human SEL-10 and SKP1. Since we proposed that SEL-10 targets int-3 for ubiquitination, we tested if the F-box domain of human SEL-10 could bind to SKP1, a component in the ubiquitination machinery, as suggested by studies on CDC4. Through co-IP assays, I demonstrated that human SEL-10 indeed binds to SKP1 through the F-box domain (Appendix D, figure 2B). This result further strengthens our model of how SEL-10 regulates Notch.

D. Phosphorylation of int-3 proteins. My data demonstrated that int-3 is a phosphorylated protein and SEL-10 binds to phosphorylated int-3 better that the unphosphorylated form (Appendix D, figure 4). This is consistent with the model that CDC4 family proteins only recognize phosphorylated target proteins and therefore, ubiquitination is usually triggered by the phosphorylation of the target protein.

Specific Aim 4: Ubiquitination of Notch Proteins.

A. Steady-state levels of int-3 proteins under proteasome inhibitor treatment. My results demonstrated that proteasome inhibitors could increase the steady state levels of Notch proteins. Treatment by lactacystin, a specific inhibitor of proteasome, could increase the steady-state levels of the C-terminal fragment of int-3 (Notch4(int-3)CHAHis in Appendix D, figure 3A) downstream of the CDC10/ankyrin repeats or a Notch1 fragment containing mainly the intracellular domain, strongly suggesting that Notch proteins can be ubiquitinated in the cell. Another proteasome inhibitor, MG132, can also stabilize a Notch1 protein containing the intracellular domain (Appendix D, figures 5A, 5B and 5C)

B. Covalent association between ubiquitin and int-3 proteins. I planed to precipitate 6Xhis tagged int-3 proteins under denaturing conditions using Ni^{2+} charged beads, then probe the precipitates with anti-ubiquitin antibody. Since the association between ubiquitin and its target protein is through covalent bond, denaturing conditions should preserve the complex between int-3 and ubiquitin while getting rid of other int-3 associated proteins, therefore, preventing false positive results and high level of background signals. However, all the anti-ubiquitin antibodies I tried proved to be less than satisfying and I could not get a repeatable result from this experiment.

Specific Aim 5: Regulations of Notch Ubiquitination and Function by SEL-10.

A. in vitro ubiquitination. We have carried out in vitro ubiquitination assays by using Notch proteins and SEL-10 immunoprecipitates from insect cells and other purified components of the ubiquitination pathway (Appendix D, figure 7). Our data indicate that SEL-10, as predicated for a CDC4 family member, interacts with other components of the ubiquitination machinery, such as SKP1, CUL1 and HRT1 (Appendix D, figure 7A). Full length SEL-10, but not a fragment of SEL-10 lacking the F-box, mediates the in vitro ubiquitination of the Int-3 protein (also named N4(int-3)HA), a C-terminal fragment of Int-3 (N4(int-3)CHAHis) and Notch1 intracellular domain (N1ICHAHis) (Appendix D, Figure 7B). When a dominant negative ubiquitin is mixed into the reaction mixture, it leads to early termination of the poly-ubiquitination of Notch protein (Appendix D, figure 7C). These results not only demonstrated that Notch proteins can be ubiquitinated, but also proved SEL-10 can mediate Notch ubiquitination.

B. Regulation of the steady-state levels of int-3 by SEL-10. My data have shown that the WD40 repeat region of SEL-10 can increase the steady-state levels of Notch proteins in a co-transfection experiment (Appendix D, figure 6). In two cases, a variant of

Notch protein is stabilized by the overexpression of the WD40 repeats of SEL-10 in a dosage dependent manner (Appendix D, figures 6A and 6B). The results are similar to that seen when Notch expressing cells were treated with lactacystin (Appendix D, figure 5), suggesting that the WD40 repeats have dominant negative effects on endogenous SEL-10.

C. Regulation of int-3 stability by SEL-10. I also addressed whether the accumulation of Notch proteins described above is caused by increased stability (half-life) of these proteins. Using pulse-chase labeling experiment, I demonstrated that the half-life of N4(int-3)C protein is prolonged by the overexpression of the WD40 repeats of SEL-10 (Appendix D, figure 6C).

D. Regulation of int-3 activity by SEL-10. I have shown in previous experiments that Notch intracellular domain can activate a luciferase reporter gene under the control of HES-1 promoter. My preliminary data (Appendix D, figures 1C and 1D, left blank) show that overexpression of the WD40 repeats of SEL-10 can enhance the Notch induced expression of luciferase, indicating that SEL-10 is able to influence Notch activity by regulating the stability of Notch.

Key Research Accomplishments

- Demonstrated that cross regulation among Notch receptors and ligands exits in endothelial cells.
- Developed reagents for the identification and functional testing of novel Notch ligands.
- Characterized the physical interactions between Notch receptors and SEL-10 at the molecular level.
- Demonstrated that SEL-10, a negative regulator of Notch is a bona fide CDC4 family protein and can mediate the ubiquitination of Notch.
- Demonstrated that SEL-10 regulates Notch activity by affecting the stability of Notch intracellular domain.

Reportable Outcomes

A. Manuscripts, abstracts and presentations

1. Wu, G., Lyapina S., Das I., Li J., Guerney M., Chui I., Deshaies R., and Kitajewski J. (2000). SEL-10, a negative regulator of Notch signaling, targets Notch for ubiquitin-mediated protein degradation. In preparation.
2. Uyttendaele H, Wu G, Roux F, Weinmaster G, Kitajewski JK. (1999). Notch4 and Jagged-1 induce microvessel differentiation of rat brain endothelial cells. Microvasuclar Research, in press.
3. Wu G., Hubbard E.J., Kitajewski J.K., and Greenwald I. (1998). Evidence for functional and physical association between Caenorhabditis elegans SEL-10, a Cdc4p-related protein, and SEL-12 presenilin. Proc Natl Acad Sci USA 95, 15787-91.
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5. Wu G., and Kitajewski J.K. (1999). F-box/WD40 repeat proteins bind to and target Notch and beta-catenin for ubiquitination. Wnt Meeting, July 17-19 1999, Stanford, California, U.S.A.
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B. Degrees obtained

1. Guangyu Wu, Doctor of Philosophy, Columbia University, May 2000.

Conclusions

Data presented in the first half of this report represent our progress in the experiments outlined in the specific aims of the original research proposal. In the past year, we developed a series of reagents that will enable us to carry out biochemical and biological studies of potential Notch ligands. We have also developed functional analysis systems in which we can quickly and easily test a candidate protein for its ability to bind and activate Notch receptors. Our observation that cross-regulations exist among different Notch pathway genes brings up an interesting angle to understand Notch ligand-receptor interaction and the potential to develop a new assay for ligand-dependent Notch activation.

In addition to the studies of Notch receptor, I also investigated the regulation of Notch signaling by *sel-10* gene. My results demonstrate that the intracellular domain of Notch can bind to the WD40 repeats of SEL-10, very likely in a phosphorylation-dependent manner. The F-box region of SEL-10 binds to other components of the ubiquitination machinery, indicating that SEL-10 negatively regulate Notch function by targeting it for ubiquitin-mediated degradation. I also provided evidence showing that Notch protein can be ubiquitinated, and its ubiquitination and stability can be regulated by SEL-10.

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Appendices

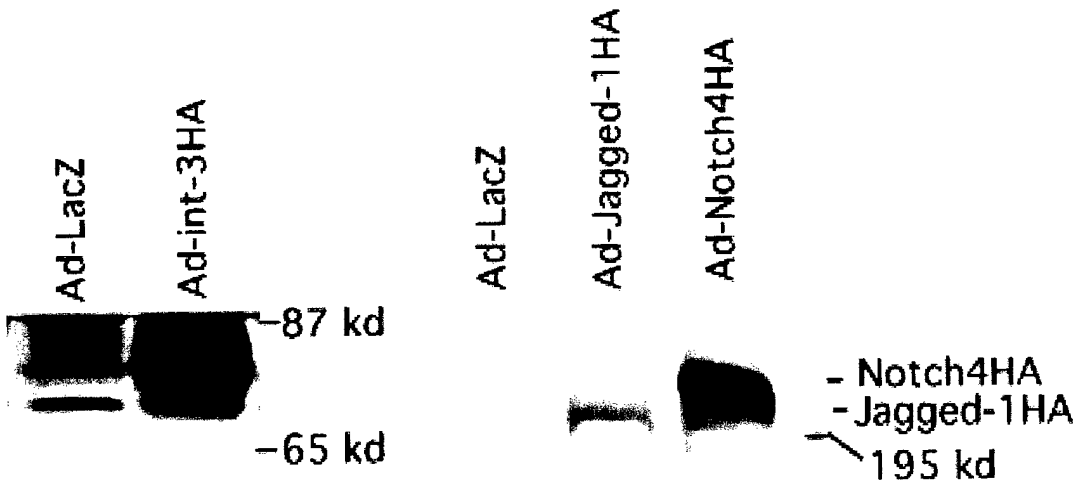
Appendix A Western blots of int-3, Notch4 and Jagged-1 proteins

Appendix B Northern blot analysis on RBE4 cells

Appendix C *sel-10*, a negative regulator of *lin-12* activity in *C. elegans*, encodes a member of the CDC4 family of proteins

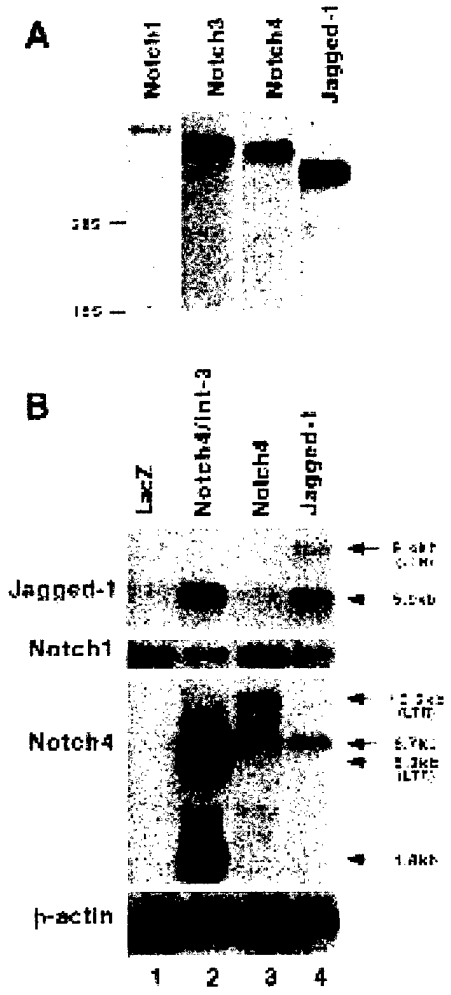
Appendix D SEL-10, a negative regulator of Notch signaling, targets Notch for ubiquitin-mediated protein degradation

Appendix A



A. HELA cells infected by adenoviruses (Multiplicity of Infection=10) carrying *LacZ, int-3HA, Notch4HA* and *Jagged-1HA* genes.

Appendix B



A. Notch1, Notch3, Notch4 and Jagged are all expressed in RBE4 cells. 40ug of total RNA from RBE4 cells hybridized to riboprobes for either *Notch1, Notch3, Notch4* or *Jagged-1*

B. Cross-regulation of Notch receptors and Ligand. 40ug of total RNA (lane 1, 3) or 20ug of total RNA (lane 2, 4) from RBE4 cells expressing LacZ, int-3, Notch4 or Jagged-1 from a CMV promoter, was hybridized to riboprobes for *Jagged-1, Notch1, Notch4* or *β-actin.* int-3 expression up-regulates the expression of endogenous Notch4 and Jagged-1. RBE4 cells stably expressing Notch receptor or ligand were generated by retroviral infection. RNA labeled with "LTR" indicates retroviral genome RNA that is transcribed from the LTR promoter.

Appendix C

sel-10, a negative regulator of *lin-12* activity in *C. elegans*, encodes a member of the CDC4 family of proteins

sel-10, a negative regulator of *lin-12* activity in *Caenorhabditis elegans*, encodes a member of the CDC4 family of proteins

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Mutations that influence *lin-12* activity in *Caenorhabditis elegans* may identify conserved factors that regulate the activity of *lin-12/Notch* proteins. We describe genetic evidence indicating that *sel-10* is a negative regulator of *lin-12/Notch*-mediated signaling in *C. elegans*. Sequence analysis shows that SEL-10 is a member of the CDC4 family of proteins and has a potential human ortholog. Coimmunoprecipitation data indicate that *C. elegans* SEL-10 complexes with LIN-12 and with murine Notch4. We propose that SEL-10 promotes the ubiquitin-mediated turnover of LIN-12/Notch proteins, and discuss potential roles for the regulation of *lin-12/Notch* activity by *sel-10* in cell fate decisions and tumorigenesis.

[*Key Words: C. elegans; lin-12;* CDC4 family; SEL-10; Notch]

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Many cell–cell interactions that specify cell fate are mediated by receptors of the LIN-12/Notch family and ligands of the Delta/Serrate/LAG-2 (DSL) family (for review, see Artavanis-Tsakonas et al. 1995). *Caenorhabditis elegans* affords an opportunity to study a simple case of lateral specification involving an interaction between two cells of the hermaphrodite gonad. These cells, named Z1.ppp and Z4.aaa, are initially equivalent in their developmental potential: each has an equal chance of becoming the anchor cell (AC), a terminally differentiated cell type that is necessary for vulval development, or a ventral uterine precursor cell (VU), which contributes descendants to the ventral uterus. In any given hermaphrodite, however, only one of these cells will become the AC, whereas the other becomes a VU (Kimble and Hirsh 1979).

Laser ablation studies have shown that this process of lateral specification, the AC/VU decision, depends on interactions between Z1.ppp and Z4.aaa (Kimble 1981; Seydoux and Greenwald 1989). Furthermore, genetic studies have indicated that *lin-12*-mediated signaling controls the AC/VU decision: if *lin-12* activity is inappropriately elevated, Z1.ppp and Z4.aaa become VUs, whereas if *lin-12* activity is reduced, Z1.ppp and Z4.aaa become ACs (Greenwald et al. 1983). Genetic mosaic analysis (Seydoux and Greenwald 1989) and reporter gene studies (Wilkinson et al. 1994) have indicated that both Z1.ppp and Z4.aaa initially express *lin-12* and *lag-2*, but that a stochastic small variation in ligand and/or receptor activity is subsequently amplified by a feedback mechanism that influences *lin-12* and *lag-2* transcription. Thus, Z1.ppp and Z4.aaa assess their relative levels of *lin-12* activity as part of the decision-making process, before either cell commits to the AC or VU fates, and the feedback mechanism ensures that only one of the two cells will become an AC and the other will become a VU.

It is striking that the receptors (LIN-12/Notch proteins), ligands (DSL proteins), and at least one downstream signaling component (CBF1/Su(H)/LAG-1; for review, see Christensen et al. 1996 and references therein) that mediate lateral specification are highly conserved in animals as distantly related as *C. elegans*, *Drosophila*, and vertebrates. Furthermore, a feedback mechanism like that first described for the AC/VU decision (Seydoux and Greenwald 1989) also exists for a Notch-mediated lateral interaction in *Drosophila* (Heitzler and Simpson 1991) and seems likely to operate in Notch-mediated lateral interactions in vertebrates (e.g., Austin et al. 1995; Chitnis et al. 1995; Washburn et al. 1997). The identification of genes that influence *lin-12* activity during the AC/VU decision may reveal other conserved factors that participate in signal transduction or regulate the activity of *lin-12/Notch* proteins.

Genetic screens based on suppression or enhancement of *lin-12* mutations have identified a number of genes

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that influence *lin-12* activity. Here we describe *sel-10*, which was first identified in a screen for suppressors of phenotypes associated with partial loss of *lin-12* activity (Sundaram and Greenwald 1993). We have found that *sel-10* acts as a negative regulator of *lin-12* signaling, and that SEL-10 is a member of the CDC4 family of F-box/WD40 repeat-containing proteins. CDC4, the most extensively studied member of this family, is a *Saccharomyces cerevisiae* protein that is involved in the ubiquitin-mediated degradation of cell cycle regulators such as SIC1 (for review, see King et al. 1996). CDC4 binds to SIC1, thereby targeting the ubiquitination machinery to this substrate (Feldman et al. 1997; Skowyra et al. 1997). Similarly, we have shown that *C. elegans* SEL-10 can interact physically with the intracellular domains of *C. elegans* LIN-12 and murine Notch4 (Robbins et al. 1992; Uyttendaele et al. 1996). We propose that SEL-10 promotes ubiquitin-mediated degradation of LIN-12/Notch proteins, and discuss potential roles for LIN-12/Notch turnover in cell fate decisions and oncogenesis.

Results

Lowering sel-10 *dosage elevates* lin-12 *activity*

Two *sel-10* alleles, *sel-10(ar28)* and *sel-10(ar41)*, were identified in a screen for suppressors of defects caused by a partial loss-of-function allele of *lin-12* (Sundaram and Greenwald 1993). These *sel-10* alleles were shown to suppress multiple defects associated with loss of *lin-12* activity, and to enhance defects associated with elevated *lin-12* activity (Sundaram and Greenwald 1993). Here, we provide evidence that *sel-10* alleles reduce *sel-10* activity, indicating that *sel-10* is a negative regulator of *lin-12* activity.

For the genetic analysis of *sel-10*, we relied on its genetic interactions with mutations in *lin-12*. We focused on two *lin-12*-mediated decisions (for review, see Greenwald 1997). One decision is made by two cells of the hermaphrodite gonad, Z1.ppp and Z4.aaa, between the AC and VU fates; normally, only one of these two cells becomes the AC, whereas the other becomes a VU (see introductory section). Eliminating *lin-12* activity causes both Z1.ppp and Z4.aaa to become ACs (the 2 AC defect), and constitutively activating LIN-12 causes both Z1.ppp and Z4.aaa to become VUs. The other decision is made by the six vulval precursor cells, between a particular vulval fate termed 2° or an alternative fate; normally, two of the six vulval precursor cells, P5.p and P7.p, adopt the 2° fate. Eliminating *lin-12* activity causes all six vulval precursor cells to adopt alternative non-2° fates, and constitutively activating LIN-12 causes all six vulval precursor cells to adopt the 2° fate. Thus, mutants in which LIN-12 is constitutively active display a 0 AC Egg-laying (Egl) defect because the absence of an AC prevents normal vulval formation; they are also Multivulva (Muv), because the descendants of each vulval precursor cell that adopts the 2° fate forms a pseudovulva.

sel-10(ar41) appears to elevate *lin-12* activity: *sel-10(ar41)* suppresses the 2 AC defect of *lin-12* hypomorphs (Sundaram and Greenwald 1993) and enhances the 0 AC defect caused by elevated *lin-12* activity (Table 1, cf. lines 1 and 2). Furthermore, the double mutant *lin-12(n379); sel-10(ar41)* displays a Muv phenotype characteristic of high *lin-12* activity that is not normally seen in *lin-12(n379)* single mutants (Table 1, lines 4,7).

The *sel-10* locus is haploinsufficient: We observed enhancement of the Muv defect (and the sterile/lethal defect) of *lin-12(n379)* hermaphrodites in *nDf42*/+ hermaphrodites (Table 1, lines 4,6).

The *sel-10(ar41)* mutation appears to reduce *sel-10* activity: the enhancement of the Muv defect of *lin-12(n379)*/+ hermaphrodites is more pronounced when *sel-10(ar41)* is placed in trans to the large deficiency *nDf42* (Table 1, lines 2,3). The greater enhancement seen in trans to a deficiency may mean that the *sel-10(ar41)* allele is a partial loss-of-function allele rather than a null allele; alternatively, *nDf42* may remove another gene that interacts with or is functionally redundant with *sel-*

Table 1. sel-10 *gene dosage analysis*

Relevant genotype	%Egl (*n*)	%Muv (*n*)	%Ste/Let (*n*)
20°			
lin-12(d)/+[a]	6 (93)	0 (93)	0 (93)
lin-12(d)/+;sel-10[b]	91 (54)	0 (54)	0 (54)
lin-12(d)/+;sel-10/Df[c]	92 (39)	15 (39)	0 (39)
15°			
lin-12(d); +[d]	86 (60)	0 (60)	0 (60)
lin-12(d);sel-10/+[e]	98 (62)	0 (62)	0 (62)
lin-12(d);+/Df[f]	89 (57)[i]	62 (74)	10 (63)
lin-12(d);sel-10[g]	100 (70)[i]	78 (197)	55 (126)
lin-12(d);sel-10/Df[h]	–	85 (34)	100 (34)[j]

Complete genotypes are as follows:
[a] *lin-12(n379)/unc-36(e251); lon-3(e2175)/him-5(e1490)*
[b] *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)*
[c] *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42*
[d] *lin-12(n379); lon-3(e2175)/him-5(e1490)*
[e] *lin-12(n379); lon-3(e2175) sel-10(ar41)/him-5(e1490)*
[f] *lin-12(n379); lon-3(e2175)/nDf42*
[g] *lin-12(n379); lon-3(e2175) sel-10(ar41)*
[h] *lin-12(n379); lon-3(e2175) sel-10(ar41)/nDf42*
Complete broods were scored by picking individual L4 animals and inspecting the plates at 24 and 48 hr for the absence of eggs on the plate (Egl) and for the presence of three or more pseudovulvae along the ventral hypodermis (Muv). Plates were then inspected after an additional three days for the presence of live progeny ["Ste/Let" refers to absence of live progeny and was, in this case, a combination of sterility (Ste) and embryonic lethality (Let)]. In some cases, broods were scored in batch for the Muv phenotype.
[i] Percent of fertile animals displaying the Egl defect.
[j] Inferred genotype: Complete broods from *lin-12(n379)/unc-36(e251);lon-3(e2175) sel-10(ar41)/nDf42)* were scored. The percentage of sterile non-Unc, non-Lon progeny (34/97 = 35%) is approximately equal to that expected for *lin-12(n379);lon-3(e2175) sel-10(ar41)/nDf42* genotypic class. Of the remaining 63 animals, 61/63 were unambiguously scored as heterozygotes in the next generation, whereas the remaining 2/63 did not have a sufficient number of progeny to score unambiguously.

10. Molecular data (see below) indicate that *sel-10(ar41)* would lead to a drastic truncation of the predicted SEL-10 protein, suggesting that *sel-10(ar41)* strongly reduces *sel-10* activity.

Elevating sel-10 *dosage lowers* lin-12 *activity*

The molecular cloning of *sel-10*(+) (see below) enabled us to examine the effect of elevated *sel-10*(+) activity, because, in general, extrachromosomal arrays formed after injecting DNA at a high concentration result in higher transgene expression (Mello et al. 1991). We found that extrachromosomal arrays containing high-copy arrays of the *sel-10* genomic region (see below) appear to lower *lin-12* activity as assayed by their effect on the AC/VU decision. There is a dramatic decrease in the proportion of *lin-12(n379)* hermaphrodites displaying the 0 AC defect in the presence of the high copy number array *arEx93* (Table 2A). In addition, the presence of the *arEx93* array enhances the 2AC defect caused by a partial loss of *lin-12* function (Table 2B). Therefore, the level of *sel-10* activity can control the level of *lin-12* activity, because increasing or decreasing the activity of *sel-10* has reciprocal effects on *lin-12* activity.

sel-10 *mutants display low penetrance defects associated with constitutive activation of* lin-12

Most *sel-10* animals appear wild type. We have observed that ~1% of *sel-10(ar41)* hermaphrodites lack an AC (data not shown). Furthermore, ~4% of *sel-10(ar41)* males display a gonad Migration (Mig) defect similar to that seen in *lin-12(d)* mutants, where it results from a failure to form the linker cell, the male counterpart of the hermaphrodite AC (Greenwald et al. 1983). In addition, we note that ~8% of *sel-10* mutant hermaphrodites are Egl even though they have an AC, and that *sel-10* males have a reduced mating efficiency that cannot be completely accounted for by the Mig defect. These additional defects may reflect the effect of increased *lin-12* activity on other cell fate decisions (Greenwald et al. 1983).

Table 2. *Increased dosage of* sel-10 *reduces* lin-12 *activity*

A. Suppression of phenotypes associated with increased *lin-12* activity	
Relevant genotype	% 0AC (n)
lin-12(d); dpy-20;Ex[sel-10(+) dpy-20(+)]	51 (47)
lin-12(d); dpy-20;Ex[dpy-20(+)]	95 (44)
B. Enhancement of phenotypes associated with reduced *lin-12* activity	
Relevant genotype	% 2AC (n)
lin-12(h); dpy-20;Ex[sel-10(+) dpy-20(+)]	97 (34)
lin-12(h); dpy-20;Ex[dpy-20(+)]	30 (40)
lin-12(+); dpy-20(e1282);Ex[sel-10(+) dpy-20(+)]	0 (87)

Complete genotypes: *lin-12(d) = lin-12(n379), lin-12(h) = lin-12(ar170), dpy-20dr(h) = dpy-20(e1282), Ex[sel-10(+) dpy-20(+)] = arEx93, Ex[dpy-20(+)] = arEx149.* (See Materials and Methods for details of strain constructions). Non-Dpy animals segregating from the strains were scored in the L3 stage for the number of anchor cells.

Table 3. *Cell autonomy of* sel-10 *function*

A. Enhancement of *lin-12(intra)*[a]		
Relevant genotype	% Egl (*n*)	% Mig (*n*)
sel-10(+);dr arEx[lin-12(intra)]	16 (88)[b]	10 (57)
sel-10(ar41);dr arEx[lin-12(intra)]	46 (136)[b]	59 (90)
B. Cell ablation[c]		
	% 0AC (*n*)	
Relevant genotype	unoperated	operated
lin-12(n379)/+; sel-10(+)	10 (57)	9 (11)
lin-12(n379)/+; sel-10(ar41)	97 (71)	83 (12)

[a]All strains also contained *him-5(e1490). dr arEx[lin-12(intra)] = arEx152* (K. Fitzgerald, pers. comm.) is an extrachromosomal array formed by microinjection (Fire 1986; Mello et al. 1991) of pRF4 [plasmid containing *rol-6(su1006)* sequence that confers a Rol phenotype onto worms carrying the array] at 100 μg/ml and pLC8 (Struhl et al. 1993).

[b]We infer that these Egl hermaphrodites lacked an AC because we scored additional hermaphrodites of relevant genotype *sel-10; arEx[lin-12(intra)]* in the L3 stage for the presence or absence of an AC and as adults for their egg-laying ability, and found that nine hermaphrodites that clearly had a single AC were non-Egl, whereas nine hermaphrodites that clearly lacked an AC were Egl.

[c]Complete genotype: *dpy-17(e164)lin-12(n379)/unc-32(e189); lon-3(e2175) sel-10(+ or ar41).* "Operated" refers to worms in which Z4 was laser ablated in the early L1 stage (when the gonad primordium consisted of four cells, Z1–Z4). Worms were then scored in the L3 stage for the presence or absence of an AC.

Cell autonomy of the sel-10 *effect on* lin-12 *activity*

Two lines of evidence suggest that *sel-10* functions cell autonomously to elevate *lin-12* activity. First, we examined the effect of reducing *sel-10* activity on the activity of the intracellular domain of LIN-12. Expression of *lin-12(intra)* causes phenotypes associated with LIN-12 activation (Struhl et al. 1993). Because LIN-12(intra) lacks the extracellular domain and, hence, is active in the absence of external signaling, an enhancement of *lin-12(intra)* activity by *sel-10* mutations would be evidence for cell autonomy of the *sel-10* effect on *lin-12* activity. We used an extrachromosomal array that contains the *lin-12(intra)* transgene and a transformation marker (see Materials and Methods); this array results in a low-penetrance *lin-12* activated phenotype (Table 3A, line 1). When this array is combined with *sel-10(ar41),* there is a dramatic increase in the proportion of hermaphrodites displaying the 0 AC-Egl defect and males displaying the Mig defect (Table 3A, line 2), suggesting that *sel-10*(+)

activity normally reduces *lin-12* function in the same cell.

We have also tested whether *sel-10* functions in the receiving end of *lin-12*-mediated cell-cell interactions by performing cell ablation experiments to remove the signaling cell, in this case Z4.aaa (Table 3B). This experiment enables different genotypes to be compared with respect to their intrinsic level of constitutive *lin-12* activity in Z1.ppp. If Z4, the precursor to Z4.aaa, is ablated in *lin-12(n379)/+* hermaphrodites, Z1.ppp usually becomes an AC, because the level of constitutive *lin-12* activity is relatively low. However, if Z4 is ablated in *lin-12(n379)/+;sel-10* hermaphrodites, Z1.ppp usually becomes a VU, suggesting that the level of constitutive *lin-12* activity is relatively high. These results suggest that *sel-10*(+) functions to reduce *lin-12* activity within the same cell, because a high level of intrinsic *lin-12* activity is seen when *sel-10* activity is reduced, even when the signaling cell is removed.

Cloning of sel-10 *by an anti-suppression assay*

sel-10 was mapped previously to an interval between *lin-25* and *unc-76* on LGV (Sundaram and Greenwald 1993). We refined the map position to a 300-kb interval between the cloned polymorphisms *arP3* and *TCPAR1* (see Materials and Methods; Fig. 1). Cosmids from the region were tested for their ability to reverse the suppression of the 2AC defect of *lin-12(ar170)* by *sel-10(ar41)* (see Materials and Methods). Arrays containing the cosmid C07E11 gave rescue in this anti-suppression assay and also reversed the enhancement of *lin-12(n379)* by *sel-10(ar41)* (data not shown). This cosmid was further subcloned and the ~8-kb fragment in pJH166 gave results similar to those seen with the entire cosmid (Fig. 1).

Molecular analysis of sel-10

The ends of pJH166 (Fig. 1) were sequenced and compared with sequence generated by the *C. elegans* genome sequencing project (Waterston et al. 1997). The entire region was found on the cosmid F55B12. A fragment from the predicted open reading frame was radiolabeled and used to screen a Northern blot and to probe a cDNA library (see Materials and Methods). Northern analysis revealed a single band of ~2.5-kb that is present in total RNA prepared from wild-type, *sel-10(ar41)* and *sel-10(ar28)* strains (data not shown). The ends of ten cDNAs from the region were sequenced and the largest cDNA was sequenced in its entirety on one strand. Verification of the 5′ end was obtained by sequencing products amplified from the cDNA library by use of trans-spliced leader sequence SL1 (Krause and Hirsh 1987) and a *sel-10*-specific sequence for primers. The splice junction of SL1 to the *sel-10* coding region occurs four bases upstream of the first predicted start codon. Figure 2 summarizes the results of the sequence analysis of *sel-10*.

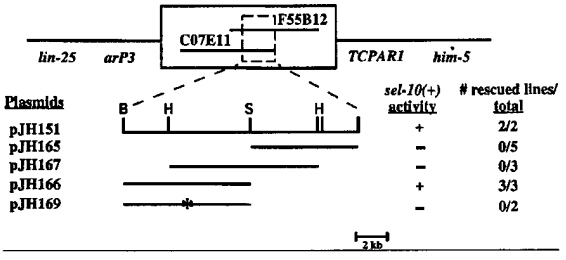

Figure 1. Molecular cloning of *sel-10*. See Materials and Methods for details of rescue assays, plasmid constructions, and molecular analysis. Genetic markers used to map *sel-10* are italicized, and two cosmids that contain *sel-10* sequences are shown in the box. The cosmid C07E11 and derivatives were tested for rescue (asterisk). pJH169 is identical to pJH166 except that it contains a stop codon in the predicted coding sequence after codon 172. Restriction sites are (B) *Bam*HI; (H) *Hin*dIII; (S) *Sal*I.

SEL-10 encodes a protein of the CDC4 family

By use of a BLAST search (Altschul et al. 1990), we found that the predicted SEL-10 protein sequence contains two previously identified amino acid sequence motifs (Fig. 3A–C). First, there is a motif (Kumar and Paietta 1995) that is now called the F-Box, after its occurrence in cyclin F (Bai et al. 1996). The F-Box motif has been implicated in protein–protein interactions, and is found in a large variety of proteins, many of which contain other recognizable motifs carboxy terminal to the F-Box (Bai et al. 1996). Second, there are seven tandem WD40 repeats, also known as β-transducin repeats, a conserved repeat of ~40 amino acids named for the common appearance of Trp-Asp (WD) at the end of the repeat (for review, see Neer et al. 1994). The crystal structure of β-transducin reveals that the seven repeats form a β propeller structure, which most likely mediates protein–protein interactions (Gaudet et al. 1996; Lambright et al. 1996; Sondek et al. 1996). There is a great deal of functional diversity among WD40 repeat-containing proteins.

The presence of an F-box amino-terminal to a set of seven WD40 motifs is the hallmark of the CDC4 family of WD40 repeat-containing proteins, indicating that SEL-10 belongs to this family. Furthermore, separate BLAST searches with just the SEL-10 F box or the SEL-10 WD40 repeats identified members of the CDC4 family as the most similar. The F-Box motif present in proteins within the CDC4 subfamily is more conserved than among other F-Box-containing proteins (Fig. 3B), and there is more extensive homology around the F-box (Kumar and Paietta 1995). In addition, the alignment of the WD40 repeats of SEL-10 and CDC4 (Fig. 3C) reveals that a given WD40 repeat is more similar between yeast and worms than are the repeats within a given species.

The CDC4 family includes proteins in fungi and vertebrates, other predicted *C. elegans* proteins (E. Kipreos, S. Gohel, and E. Hedgecock, pers. comm.; E.J.A. Hubbard, unpubl.), and several mammalian proteins (Kumar and Paietta 1995; Bai et al. 1996). The best studied member of this family, *Sacharomyces cerevisiae* CDC4, targets SIC1 and certain G_1 cyclins for degradation (for re-

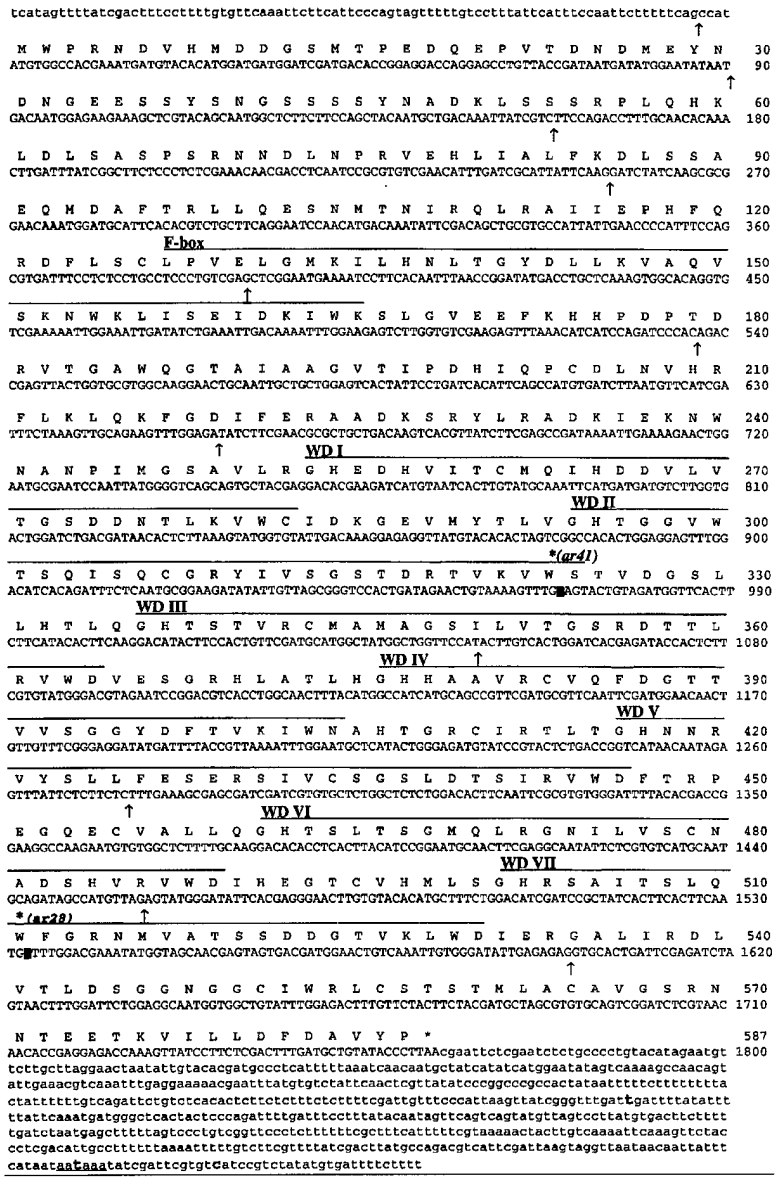

Figure 2. cDNA sequence and predicted protein product of *sel-10.* Splice junctions are indicated by arrows below the DNA sequence. The first arrow indicates the SL1 splice junction. The F-Box (Kumar and Paietta 1995, Bai et al. 1996) and the WD40 repeats are overlined and labeled in the Figure. The lesions in *sel-10(ar41)* and *sel-10(ar28)* are indicated with reverse contrast letters in the nucleotide sequence and a bold asterisk above the amino acid; both are G to A transitions resulting in W to stop codon changes in the amino acid sequence at residues 323 and 511, respectively. The cDNA termination codon is marked with an asterisk. A sequence conforming to the consensus polyadenylation signal sequence is underlined, and sites of polyA attachment are marked in bold. Two independent cDNAs contained polyA 14 nucleotides downstream of this signal; two alternative sites of attachment were also observed. Sequence information has been submitted to GenBank (accession no. AF020788).

view, see King et al. 1996). Not all CDC4 family members, however, are cell cycle regulators. For example, there are proteins that negatively regulate sulfur metabolism from *S. cerevisiae, Neurospora crassa,* and *Aspergillus nidulans* (Natorff et al. 1993; Kumar and Paietta 1995; Thomas et al. 1995).

A recent database search has revealed potential human and rat SEL-10 orthologs (Fig. 3D). The limited sequence information available to date for the human gene begins in the sixth WD40 repeat and extends to the carboxyl terminus, and in this region, there is 60% sequence identity. BLAST searches of the available databases with the SEL-10 sequence carboxy-terminal to the WD40 repeats have not identified any other highly conserved sequences.

sel-10 *mutations truncate the SEL-10 protein*

Sequence analysis of *sel-10* mutations supports the genetic evidence suggesting that they strongly reduce *sel-10* activity. The sequence alterations caused by *sel-10(ar41)* and *sel-10(ar28)* were determined by direct sequencing of amplified genomic DNA products (see Materials and Methods). Both alleles are nonsense mu-

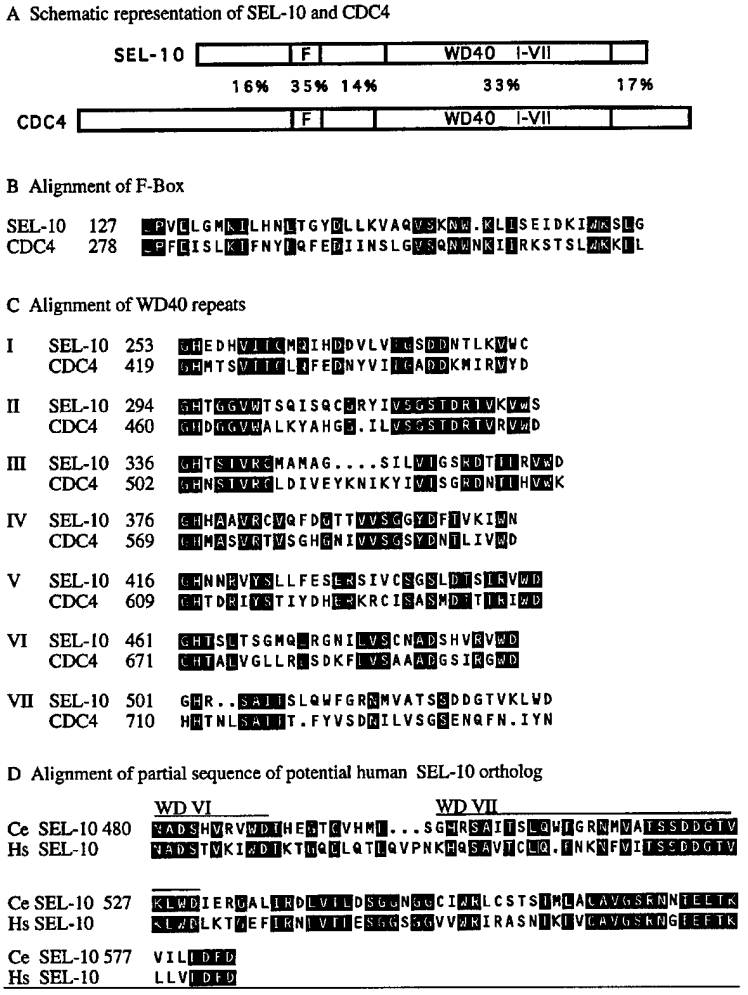

Figure 3. SEL-10 is a member of the CDC4 family of F-box/WD40-repeat proteins. The CDC4 sequence (accession no. X05625) is from GenBank. There are other potential CDC4 family members in the database; limited sequence data for a potential SEL-10 ortholog is given in part D. For further discussion of the CDC4 family, see Kumar and Paietta (1995) and Bai et al. (1996). Reverse contrast letters indicate amino acid identity. (*A*) Schematic depiction of SEL-10 and CDC4, drawn to scale. The percentage of identical amino acids in each region is indicated. Members of the CDC4 family all have this general organization, with some variability in the length and sequence of their amino and carboxyl termini. (*B*) Alignment of SEL-10 and CDC4 F-Boxes. (*C*) Alignment of WD40 repeats from SEL-10 and CDC4. (*D*) Alignment of SEL-10 and a potential human ortholog. Partial sequence of a human cDNA encoding a sequence highly similar to the carboxyl terminus of SEL-10 was obtained from Genbank (accession no. H22962) and was extended into the WD40 repeats by direct sequencing of clone ym50h08.s1 (G. Wu and J. Kitajewski, unpubl.). The predicted amino acid sequence encoded by the available human cDNA sequence is shown. The database also contains partial sequence information for a rat cDNA (Genbank accession no. H34371) that is predicted to encode a peptide that is 100% identical to the last 35 amino acids of the available human sequence (data not shown).

tations at nucleotide positions 969 and 1533, respectively (see Fig. 2), resulting in truncated predicted proteins.

sel-10(ar41) removes the carboxy-terminal half of the protein, including five of the seven WD40 repeats. This observation suggests that *sel-10(ar41)* is likely to result in a nonfunctional SEL-10 protein. It is unlikely that the two WD40 repeats that remain in this protein are functional because there are no known WD40-repeat-containing proteins with only two repeats (Neer et al. 1994). Furthermore, the crystal structure of β transducin reveals that the seven repeats form a β propeller structure that would not be complete in the absence of five of the seven repeats (Sondek et al. 1996). Finally, comparable mutations in another *C. elegans* CDC4 subfamily protein, LIN-23, behave like molecular null alleles (E.T. Kipreos, S.P. Gohel, and E.M. Hedgecock, pers. comm.).

C. elegans *SEL-10 physically interacts with LIN-12(intra) and murine Notch4(int3)*

We probed for potential interactions between SEL-10 and the intracellular domains of LIN-12/Notch proteins, specifically LIN-12(intra), the intact intracellular domain (Struhl et al. 1993) and Notch4(int3), the intact intracellular domain with some additional sequences produced by the *int3* mutation (Robbins et al. 1992; Uyttendaele et al. 1996). We initially used the yeast two-hybrid system (Fields and Song 1989) and our preliminary results suggested that SEL-10 physically interacted with the *C. elegans* LIN-12 intracellular domain, the *C. elegans* GLP-1 intracellular domain (GLP-1 is another *C. elegans* LIN-12/Notch protein; see Yochem and Greenwald 1989), and the mouse Notch4(int3) intracellular domain (data not shown).

To examine further whether SEL-10 binds LIN-12/Notch proteins, we carried out coimmunoprecipitation experiments by use of transfected mammalian cells (Fig. 4). 293T (Bosc23) cells (human embryonic kidney cells) were transiently transfected with hemagglutinin HA-tagged LIN-12(intra) and/or myc-tagged SEL-10 (see Materials and Methods). Transfected cells were lysed and LIN-12(intra)HA was precipitated with anti-HA antibod-

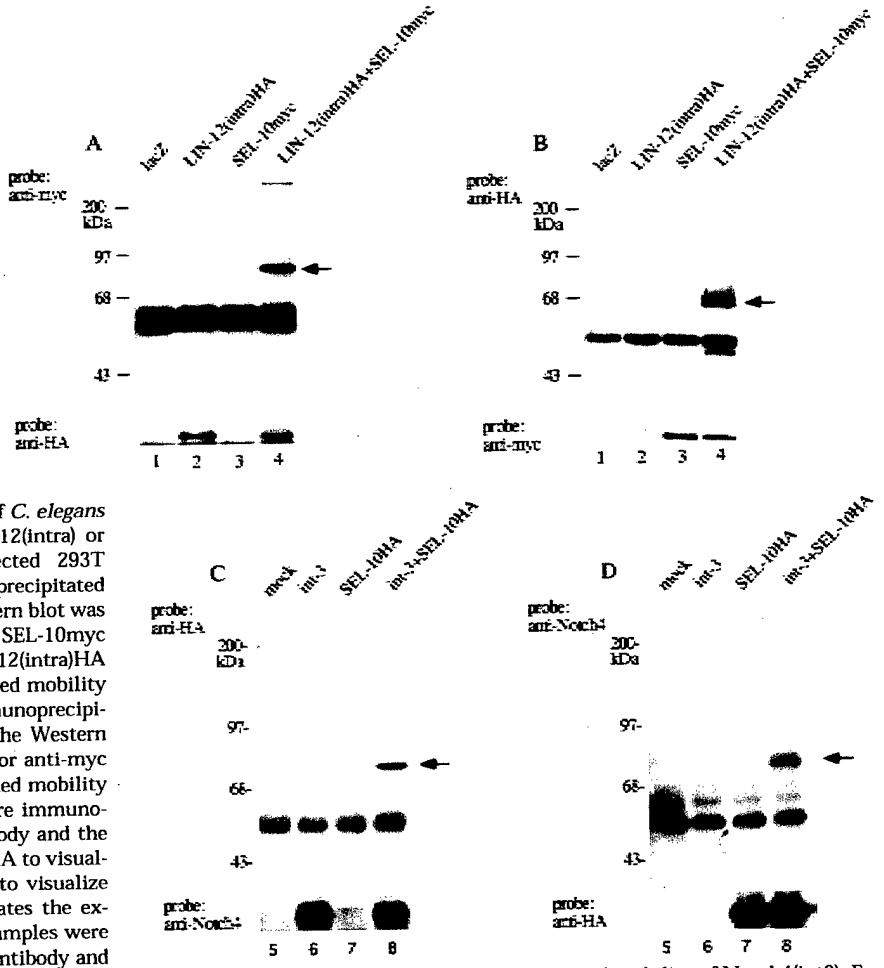

Figure 4. Coimmunoprecipitation of *C. elegans* SEL-10 with either *C. elegans* LIN-12(intra) or murine Notch4(int3) from transfected 293T cells. (*A*) Samples were immunoprecipitated with anti-HA antibody and the Western blot was probed with anti-myc to visualize SEL-10myc (*top*) or anti-HA to visualize LIN-12(intra)HA (*bottom*). Arrow indicates the expected mobility of SEL-10myc. (*B*) Samples were immunoprecipitated with anti-myc antibody and the Western blot was probed with anti-HA (*top*) or anti-myc (*bottom*). Arrow indicates the expected mobility of LIN-12(intra)HA. (*C*) Samples were immunoprecipitated with anti-Notch4 antibody and the Western blot was probed with anti-HA to visualize SEL-10HA (*top*) or anti-Notch4 to visualize Notch4(int3) (*bottom*). Arrow indicates the expected mobility of SEL-10HA. (*D*) Samples were immunoprecipitated with anti-HA antibody and the Western blot was probed with anti-Notch4 (*top*) or anti-HA (*bottom*). Arrow indicates the expected mobility of Notch4(int3). For details, see Materials and Methods. (Lane *1*) pQNClacZ; (lane *2*) pQNClin-12(intra)HA + pQNClacZ; (lane *3*) pQNClacZ + pQNCsel-10myc; (lane *4*) pQNClin-12(intra)HA + pQNCsel-10myc; (lane *5*) mock transfected cells; (lane *6*) pLNCint3 + pQNCX; (lane *7*) pLNCX + pQNCsel-10HA; (lane *8*) pLNCint3 + pQNCsel-10HA.

ies (Fig. 4A) or, alternatively, SEL-10myc was immunoprecipitated with anti-myc antibodies (Fig. 4B). The immunoprecipitates were subjected to immunoblot analysis to identify bound proteins, and probed with anti-myc or anti-HA antibodies as indicated. Under both conditions, the immunoprecipitates were found to contain both LIN-12HA and SEL-10myc. This result suggests that SEL-10 and LIN-12 are able to interact physically, either directly or in a complex.

We also examined whether the *C. elegans* SEL-10 protein would interact with the murine Notch4(int-3) protein. Cells were transfected with Notch4(int-3) and/or SEL-10HA, and immunoprecipitation was performed with cell lysates by use of either anti-Notch4 antibodies (Fig. 4C) or anti-HA antibodies (Fig. 4D). Immunoblot analysis showed that the immunoprecipitates contained a complex of Notch4(int-3) and SEL-10 proteins; thus, SEL-10 is able to complex with Notch4(int-3) protein.

The observations that *sel-10* negatively regulates *lin-12* activity, that SEL-10 resembles CDC4, and that SEL-10 physically interacts with LIN-12, taken together, strongly suggest that SEL-10 functions biochemically like CDC4 to promote LIN-12 turnover. We have attempted to examine the effect of coexpressing *C. elegans* SEL-10 on the steady-state levels and ubiquitination of Notch4(int-3) and observed a modest decrease in the steady state level (data not shown); however, at this time the mechanism underlying this decrease is not clear. Furthermore, there appears to be polyubiquitination of Notch4(int-3) even in the absence of transfected SEL-10, perhaps caused by the activity of an endogenous mammalian *sel-10*-like gene (data not shown).

Discussion

In this paper, we have presented genetic evidence indi-

cating that *sel-10* is a negative regulator of *lin-12*-mediated signaling in *C. elegans*. Mutations that lower *sel-10* activity, elevate *lin-12* activity, and increasing *sel-10* dosage lowers *lin-12* activity, suggesting that the level of *sel-10* activity can influence *lin-12* activity. Furthermore, *sel-10* appears to act in the same cell as *lin-12*.

We propose that the mechanism by which *sel-10* affects *lin-12* activity may be by controlling LIN-12/Notch protein levels. Sequence analysis indicates that SEL-10 is related to the *S. cerevisiae* protein CDC4, which promotes the ubiquitin-dependent degradation of cell cycle regulators (for review, see King et al. 1996). CDC4 complexes with its substrates (Feldman et al. 1997; Skowyra et al. 1997), and we have found that *C. elegans* SEL-10 complexes with the intracellular domain of LIN-12. Proteins related to SEL-10 exist in mammals, and *C. elegans* SEL-10 physically complexes with Notch4(int3), the intracellular domain of murine Notch4. These observations suggest that negative regulation of LIN-12/Notch by SEL-10 may be an evolutionarily conserved feature.

SEL-10 may target LIN-12/Notch proteins for ubiquitin-mediated degradation

The attachment of ubiquitin to substrates involves a series of protein complexes. Ubiquitin is activated by linkage to an E1 ubiquitin activating enzyme, then transferred to an E2 ubiquitin conjugating enzyme. Some ubiquitination events also require the action of a third complex, termed E3. It is thought that E3 complexes may contribute to substrate specificity (for review, see Ciechanover 1994; King et al. 1996). The *S. cerevisiae* protein CDC4 may function in an E3 complex. *CDC4* is one of a group of genes that also includes *CDC34, CDC53,* and *SKP1;* together, the proteins encoded by these genes directly regulate the level of the cyclin-dependent kinase inhibitor SIC1, which must be destroyed for progression from G_1 to S phase. CDC34 is an E2 ubiquitin conjugating enzyme (Goebl et al. 1988), and the current view is that CDC4, CDC53, and SKP1 function as an E3 complex (Bai et al. 1996; Mathias et al. 1996). On the basis of our analysis of *sel-10* and the data for *CDC4*, we propose that SEL-10 functions as part of an E3 complex to target the intracellular domains of LIN-12/Notch proteins for ubiquitin-dependent degradation.

The carboxy-terminal region of all LIN-12/Notch proteins contain PEST sequences and one or more lysines immediately carboxy-terminal to the cdc10/SWI6 motifs. The presence of PEST sequences often indicates that ubiquitin-mediated turnover occurs, although the two are not necessarily strictly correlated (Rogers et al. 1986). With respect to the CDC34/CDC4-mediated events, it appears that the PEST sequences couple phosphorylation of the substrate to attachment of ubiquitin to lysine residues (for review, see King et al. 1996). The PEST sequence found in the carboxy-terminal region of all LIN-12/Notch proteins, however, may not be required for SEL-10-mediated degradation of LIN-12/Notch proteins, because *sel-10* mutations can still enhance the gain-of-function phenotype of a *glp-1* allele that is truncated prior to the PEST sequence (E.J.A. Hubbard, unpubl.).

An important issue to consider in the context of SEL-10 as a component of an E3 complex is its specificity for LIN-12/Notch proteins. The available *C. elegans* genetic data suggest that *sel-10* is an allele-nonspecific, gene-specific suppressor of *lin-12,* supporting a role for SEL-10 specifically in regulating the activity of LIN-12, or perhaps a small set of proteins including LIN-12. Allele-nonspecificity is indicated by the observation that mutations in *sel-10* suppress/enhance all *lin-12* alleles tested (Sundaram and Greenwald 1993; this report; E.J.A. Hubbard, unpubl.). Gene-specificity is suggested by the fact that mutations in *sel-10* have not been identified in numerous screens in many laboratories for suppressors of hypomorphic mutations in genes encoding proteins other than LIN-12/Notch proteins; furthermore, we have not observed suppression of various marker mutations used in routine strain constructions or of hypomorphic alleles of several other genes encoding receptor proteins (E.J.A. Hubbard, unpubl.).

The available genetic data also suggest that *sel-10* activity does not regulate cell cycle progression, a possibility raised by the phenotype of *cdc4* mutants. Mutations in *cul-1,* a *C. elegans* gene related to *S. cerevisiae CDC53,* cause hyperplasia of larval blast cells, suggesting that *cul-1* regulates cell-cycle progression (Kipreos et al. 1996). We have not seen any evidence that hyperplasia occurs in *sel-10(ar41)* mutants (E.J.A. Hubbard, unpubl.). Because *sel-10(ar41)* mutants have little or no *sel-10* activity (see Results), we think that it is unlikely that *sel-10* is involved in cell cycle regulation per se, unless there is another functionally redundant gene that masks cell cycle involvement of *sel-10.* In contrast, mutations in another *CDC4* related gene, *lin-23,* do cause hyperplasia, consistent with a role for *lin-23* in the regulation of cell cycle progression (E.T. Kipreos, S.P. Gohel, and E.M. Hedgecock, pers. comm.).

Potential roles for LIN-12/Notch protein downregulation or turnover in cell fate decisions

Proteolysis of LIN-12/Notch proteins might occur in response to ligand binding or occur constitutively. For a variety of cell surface receptors, ligand-induced polyubiquitination appears to be a mechanism for down-regulation (for review, see Ciechanover and Schwartz 1994). Although we do not, at this time, have direct evidence for ligand-induced ubiquitination of LIN-12/Notch receptors, we note that LIN-12(intra), which genetically and physically interacts with SEL-10, behaves like an activated receptor (Struhl et al. 1993). Alternatively, SEL-10 may target any form of LIN-12/Notch (activated or unactivated) for degradation. Although constitutive turnover is not strictly a mechanism for controlling receptor activity per se, it would, in effect, sensitize the system to other control mechanisms such as transcriptional regulation (see below) by generally reducing the amount of LIN-12.

Constitutive turnover or ligand-induced down-regulation of LIN-12/Notch proteins may be important for cell

fate decisions to occur normally. We can best illustrate potential roles for turnover or down-regulation with the AC/VU decision (Seydoux and Greenwald 1989; Wilkinson et al. 1994) as an example. Initially, Z1.ppp and Z4.aaa have equal signaling and receiving potentials; ligand (LAG-2) and receptor (LIN-12) may interact, but signaling activity is below a critical threshold. SEL-10-mediated turnover or down-regulation of LIN-12 might prevent this initial signaling from causing both cells to achieve the threshold value of effector activity. Thus, one possible role for receptor turnover or down-regulation would be to limit the output from a single ligand-receptor interaction.

Another potential role for receptor turnover or down-regulation is in enhancing differences in *lin-12* activity between interacting cells. During the AC/VU decision, a small stochastic difference between the two cells is amplified by a feedback mechanism (Seydoux and Greenwald 1989). The feedback mechanism appears to involve differential transcription of ligand and receptor genes: activation of LIN-12 appears to repress transcription of *lag-2* and to stimulate transcription of *lin-12* (Wilkinson et al. 1994). The feedback mechanism ensures that the cell with higher *lin-12* activity becomes the VU whereas the cell with lower *lin-12* activity becomes the AC. Down-regulation of LIN-12 would be necessary for differences in transcription to be manifest. In the absence of down-regulation, signaling from activated receptor would persist, masking the effects of differential transcription. Indeed, this situation is analogous to the role of ubiquitin-mediated degradation of G_1 cyclins (King et al. 1996).

Turnover of LIN-12/Notch proteins may play different or additional roles in other cell fate decisions. For example, in *Drosophila* eye development, Notch appears to be utilized for sequential cell fate decisions (Cagan and Ready 1989), which would seem to necessitate clearance of activated Notch after each decision so that a new assessment of *Notch* activity can be made. Furthermore, it is also conceivable that for some LIN-12/Notch mediated decisions, the cell fate adopted may depend on the intensity of signal, as has been seen for receptors for gradient morphogens (e.g., Nellen et al. 1996). If any LIN-12/Notch-mediated decisions do display such dosage sensitivity, it is likely that they would depend on rapid turnover of activated receptor complexes so that the correct threshold value is read.

The fact that most *sel-10(ar41)* individuals are phenotypically wild-type, with only a small proportion displaying phenotypes associated with LIN-12 activation, may be explained in this context if there is a redundant gene product or regulatory mechanism. There are other CDC4 related genes in the *C. elegans* genome (E.J.A. Hubbard, unpubl.). Furthermore, there may be other mechanisms for degrading LIN-12. For example, *sel-1*, another negative regulator of *lin-12* activity, may also be involved in LIN-12 turnover (Grant and Greenwald 1997), but because SEL-1 is an extracytosolic protein, it is not likely to be directly involved in the ubiquitination of the intracellular domain of LIN-12.

Potential roles for sel-10 *in oncogenesis*

Mammalian tumors induced by expression of Notch4(int3) or other truncated forms of Notch largely consisting of the intact intracellular domain are thought to result from constitutive Notch activity (Ellisen et al. 1991; Robbins et al. 1992; Uyttendaele et al. 1996). Because SEL-10 down-regulates Notch activity, it may act to restrain either normal or oncogenic functions of activated Notch, and, hence, suppress cell growth. If so, loss-of-function mutations in vertebrate *sel-10* could contribute to oncogenesis mediated by Notch by elevating the level of Notch protein. For instance, human T acute lymphoblastic leukemias, which in the majority of cases do not contain oncogenic Notch alterations (Drexler et al. 1995), and human breast tumors, which thus far have not been reported to contain oncogenic Notch alterations, may carry mutations in other proteins that influence *Notch* activity, such as *sel-10* homologs.

Materials and methods

General methods and strains

General methods are described by Brenner (1974). The wild-type parent for all strains was *C. elegans* var. Bristol strain N2. Mapping experiments utilized the Bristol/Bergerac congenic strain GS352, in which the region between *rol-4* and *par-1* of Bristol was replaced with the corresponding region from the Bergerac strain BO (Tuck and Greenwald 1996). Strains were grown at 20°C unless otherwise noted. Mutations used are described in Hodgkin (1997); additional references for critical alleles are also given. Note that all genetic constructions containing *sel-10* used a *sel-10* chromosome that does not have the linked modifier mutation *arX* (Sundaram and Greenwald 1993). LGIII: *dpy-17(e164), unc-36(e251),* and *unc-32(e189); lin-12(ar170)* (Hubbard et al. 1996; E.J.A. Hubbard, unpubl.); *lin-12(n379)* (Greenwald et al. 1983). LGIV: *dpy-20(e1282).* LGV: *nDf42* (M. Hengartner and H.R. Horvitz, pers. comm.), *lon-3(e2175), rol-4(sc8), sel-10(ar41)* (Sundaram and Greenwald 1993), *him-5(e1490), unc-76(e911).*

Mapping of the sel-10 *locus*

sel-10 had been genetically mapped between *lin-25* and *unc-76* V (Sundaram and Greenwald 1993) and ~0.2 MU to the left of *him-5* (data not shown). We mapped *sel-10* between *arP3* and *TCPAR1* by identifying Rol Him non-Unc recombinants from heterozygotes of the genotype *rol-4 BO unc-76/lon-3 sel-10 him-5* constructed with the strain GS352. Fifty independent recombinants were analyzed by Southern blot hybridization for the presence of *arP3* and *TCPAR1* (Tuck and Greenwald 1996), and each recombinant strain was tested for the presence of *sel-10(ar41)* by crossing into *lin-12(n379)* and scoring for the Muv phenotype. Mapping data can be found in ACeDB (Edgley et al. 1997).

sel-10 *cloning by antisuppression assay*

sel-10(ar41) partially suppresses the 2AC defect caused by *lin-12(ar170):* at 25°C, ~80% of *lin-12(ar170)* animals have 2AC whereas ~25% of *lin-12(ar170); sel-10(ar41)* animals have 2AC. We used reversal of suppression as the basis of assessing *sel-10*(+) activity of microinjected DNAs. Transgenic lines were generated by microinjecting the germ lines of *lin-12(ar170);*

dpy-20(e1282);sel-10(ar41) him-5(e1490) hermaphrodites with cosmid or plasmid DNA (Mello et al. 1991) at a concentration of 5 μg/ml, along with the *dpy-20*(+) transformation marker DNA at 10 μg/ml (plasmid pMH86; Han and Sternberg 1991) and carrier Bluescript DNA (Stratagene) at 90 μg/ml. Synchronous populations were obtained by allowing groups of transgenic hermaphrodites to lay eggs at 20°C for 1- to 2-hr and tranferring the eggs to 25°C. The non-Dpy L3 hermaphrodites were then scored for the number of anchor cells. The injected tester DNA was considered to contain *sel-10*(+) sequences if >50% of the non-Dpy animals had 2AC. Typically, 60%–80% of hermaphrodites had 2AC in these rescued lines. Some arrays scored as having *sel-10*(+) activity were subjected to a second test, the ability to reverse the Muv phenotype of *lin-12(n379);sel-10 (ar41)*. Initial rescue was obtained with a pool of seven overlapping cosmids from the region (each at 5 μg/ml), then with the single cosmid C07E11, and then with plasmids derived from C07E11, as shown in Figure 1.

Plasmids containing sel-10 *genomic sequences*

pJH151 was constructed by digesting cosmid C07E11 with *Bam*HI and ligating the 15-kb fragment to Bluescript KS+ (Stratagene). pJH166 was constructed by ligating an 8-kb *Pst*I–*Sal*I fragment from pJH151 into Bluescript KS+. The *Pst*I site was from the vector, whereas the *Sal*I site is from the genomic sequences. The ~9-kb *Sal*I fragment was removed from pJH151 to form pJH165, and pJH167 was made by ligation of the internal *Hin*dIII fragment of pJH151 into Bluescript. To construct pJH169, pJH166 was cut with *Pme*I and a linker containing an *Nhe*I site with a stop codon in all frames (NEB 1060) was inserted, creating a stop codon after amino acid 172 in the SEL-10 sequence.

sel-10 *overexpression*

arEx93 was generated by microinjecting *dpy-20* hermaphrodites with pJH166 [*sel-10*(+)] at a concentration of 100 μg/ml and pMH86 [*dpy-20*(+)] at 10 μg/ml. Strains carrying this array segregate sterile animals as well as fertile animals; the basis for the sterility has not been established. Many of the fertile animals display a leaky Egl phenotype similar to that observed in certain *lin-12* hypomorphic mutants. Qualitatively similar results were observed with other lines at this concentration and with lines established by use of pJH166 at 50 μg/ml (data not shown).

The control array *arEx149* was established by microinjecting *unc-32; dpy-20* hermaphrodites with pMH86 at 10 μg/ml, and Bluescript DNA at 90 μg/ml into *unc-32; dpy-20* animals.

Molecular analysis of sel-10

Standard methods were used for the manipulation of recombinant DNA (Sambrook et al. 1989). *sel-10*(+) cDNAs were obtained by screening ~100,000 pfu from a phage library kindly provided by R. Barstead (Barstead and Waterston 1989). Ten positive plaques were purified by two subsequent rounds of screening with a radiolabeled fragment from pJH166 (~8-kb *Bam*HI–*Sal*I fragment) as a probe. cDNA 1A, the longest cDNA obtained, was sequenced in its entirety on one strand and compared with genomic sequence from the genome project with GENEFINDER (Waterston et al. 1997). The sequence of the cDNA 1A differed from the GENEFINDER prediction in the location of the junction between the second and third exons and in the predicted 3′ end. Four of the cDNAs were polyadenylated at their 3′ ends (one 294, one 581, and the other two 601 bases after the predicted stop codon). Of these, only the last two were in the context of a conserved polyadenylation signal. The 5′-most cDNA end was located in codon 1 (cDNA 8 begins at G of the first ATG), but a PCR product was amplified from DNA prepared from the same cDNA library (Barstead and Waterston 1989; C. Dong, pers. comm.) contained the SL1 spliced leader at the predicted sequence 4 bases 5′ of the first ATG. The 22 base SL1 sequence and a primer straddling the 5th and 6th exons were used for the 5′ end amplification.

Sequence analysis

Standard techniques were used to obtain sequence of the 1A cDNA (Sambrook et al. 1989). The lesions associated with the *sel-10(ar41)* and *sel-10(ar28)* mutations were found by direct sequencing of two PCR products from single-stranded templates (Allard et al. 1991; Kaltenboeck et al. 1992), by use of internal primers to cover the entire region. One small segment was subcloned and sequenced (from two independent reactions each), as the sequence from this region was not easily generated by use of the direct method. Sequence comparisons and alignments were obtained by use of Blast (Altschul et al. 1990) through the NCBI web site and GCG (version 8, Devereux et al. 1984) programs.

Plasmids for cell culture experiments

Plasmids used in the transient transfection experiments were constructed in pLNCX (Miller et al. 1989) or pQNCX (Qingyou Yan and J.K., unpubl.), vectors that drive gene expression under the control of a CMV promoter. pQNClacZ contains the bacterial *lacZ* gene; pQNClin-12(intra)HA encodes a protein with a methionine-containing hemagglutinin epitope (Wilson et al. 1984) fused in frame amino-terminal to LIN-12(intra) at amino acid 939. pLNCint-3 contains cDNA corresponding to the *Notch4* region expressed in the *int3* insertion, beginning at amino acid 1411; the Notch4(int3) protein includes the entire intracellular domain of Notch4 and additional sequences (Uyttendaele et al. 1996). pQNCsel-10myc (pJH186) encodes a protein with six myc epitope tags (Roth et al. 1991) fused in frame to cDNA 1A at amino acid 13 of SEL-10. pQNCsel-10HA (pJH184) encodes a protein with a methionine-containing hemagglutinin epitope from pACT2 (Durfee et al. 1993) fused in frame (along with a short stretch of polylinker) to cDNA 1A at amino acid 13.

Transfection, immunoprecipitations, and Western blot analysis

293T (Bosc23) cells (Pear et al. 1993) were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS). A confluent plate of cells was split 1:3 the day prior to transfection. For one 60-mm plate of cells, 4 μg of each plasmid DNA was transfected by the calcium phosphate precipitation method. The total amount of DNA was kept constant by supplementation with vector DNA or *lacZ*-containing plasmids.

Two days after transfection, cells were harvested and lysed in TENT buffer (50 mM Tris-HCl at pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing protease inhibitors (2 μg/ml aprotinin, 2 μg/ml of leupeptin, 2 μg/ml of pepstatin, 0.5 mM PMSF). Lysates were clarified by centrifugation at 10,000*g* for 10 min and protein content was determined with the BioRad Protein determination kit and samples were normalized for protein content. Extracts were precleared with Sepharose CL-4B beads, incubated with antibodies (3 μl of anti-Notch4 antiserum, 50 μl of 12CA5 anti-HA supernatant, or 200 μl of 9E10 anti-myc supernatant) for 6 hr at 4°C, then incubated with 40 μl of 50%

slurry of protein A-Sepharose for 1 hr at 4°C. The protein A-Sepharose beads were washed with TENT buffer three times by vortexing for 10 min, beads were boiled in 30 µl 1× protein loading buffer, and then electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The blot was blocked overnight at 4°C with TBST (10 mM Tris at pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 1% bovine serum albumin (TBST-BSA). The blot was then incubated with 1° antibody diluted (1:2,000 anti-Notch4; 1:50 for 12CA5; 1:10 for 9E10) in TBST-BSA for 1 hr, washed three times for 5 min each with TBST, and incubated with 2° antibody in TBST-BSA for 1 hr. After three washes, the signal was visualized by chemiluminescence (Amersham, ECL).

The anti-Notch4 antiserum (G. Wu and J. Kitajenski, unpubl.) is directed against the carboxy-terminal region of Notch4 (residues 1788–1964) (Uyttendaele et al. 1996). 12CA5 anti-HA antibody was obtained from Berkeley Antibody Co., Richmond, CA. 9E10 anti-myc antibody was prepared from culture supernatants of the 9E10 hybridoma (Evan et al. 1985).

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Appendix D

SEL-10, a negative regulator of Notch signaling, targets Notch for ubiquitin-mediated protein degradation

SEL-10, a Negative Regulator of Notch Signaling, Targets Notch for Ubiquitin-mediated Protein Degradation

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Running Title: SEL-10 targets Notch for Ubiquitin-mediated Turnover

SUMMARY

Notch receptors play important roles in both normal animal development and pathogenesis. We show that the CDC4 family protein SEL-10 negatively regulates Notch receptor activity by targeting the intracellular domain of Notch receptors for ubiquitin-mediated protein degradation. The WD40 repeats of SEL-10 interact with the C-terminal domain of Notch receptors. SEL-10 binds preferentially to a phosphorylated form of Notch4. Using an *in vitro* assay, SEL-10 protein can mediate the ubiquitination of Notch proteins. Blocking endogenous SEL-10 activity by over-expression of a dominant-negative form of SEL-10 with only the WD40 repeats leads to stabilization of the intracellular domain of Notch, as evidenced by increased steady-state levels, prolonged half-life and up-regulated Notch induced reporter activation.

INTRODUCTION

Notch/LIN-12 receptors regulate cell fate decisions during both normal animal development and pathogenesis. For example, in *C. elegans*, LIN-12 activities ensure that only one of the two undifferentiated gonadal cells develop into an anchor cell (AC), while the other cell become a ventral uterine precursor cell (VU) (Greenwald, 1998). In humans *Notch* genes have been linked to several pathologies, including cancer (Luo et al., 1997), vascular integrity (Joutel et al., 1996), and possibly schizophrenia (Wei and Hemmings, 2000).

Notch/LIN-12 signaling pathway is activated when ligand-receptor interaction induces a proteolytic cleavage event that releases the intracellular domain of the receptor from cell membrane (Mumm et al., 2000; Schroeter et al., 1998; Struhl and Greenwald, 1999). As the signaling module of a Notch/LIN-12 receptor, the intracellular domain is then translocated into the nucleus and directly involved in modulating gene expression (Struhl and Adachi, 1998). The nuclear activity of Notch/LIN-12 receptors at least partially relies on the interaction between the intracellular domain of a Notch/LIN-12 receptor and a transcription factor known as Suppressor of Hairless (Su[H]) in *Drosophila*(Fortini and Artavanis-Tsakonas, 1994) , Lag-1 in *C. elegans*, and CBF-1 or RBP-J_ in mammals. The complex of Su(H) and Notch/LIN-12 intracellular domain functions as a transcriptional activator and up-regulates genes containing the regulatory sequence recognized by the Su(H) DNA binding domain. Much less knowledge is available regarding the subsequent down-regulation of Notch signaling.

The *C. elegans* gene *SEL-10* was first identified in a genetic screen as a negative regulator of the Notch/LIN-12 signaling pathway (Sundaram and Greenwald, 1993).

Molecular cloning and sequence analyses reveal that SEL-10 is a homologue of the yeast protein CDC4 (Hubbard et al., 1997). Members of the CDC4 protein family are characterized by an F-box domain (Winston et al., 1999a) and seven WD40 repeats, both protein-protein interaction motifs. In previous studies, CDC4 family proteins have been shown to mediate target protein ubiquitination and degradation. Specifically, the WD40 repeats of a CDC4 family protein bind to the target protein in a phosphorylation-dependent manner, while the F-box domain interacts with other proteins in the ubiquitination machinery, such as SKP1 (Deshaies, 1999; Feldman et al., 1997; King et al., 1996). Based on such knowledge, it has been proposed that SEL-10 negatively regulates Notch/LIN-12 signaling by targeting the intracellular domain of Notch/LIN-12 receptors for ubiquitin-mediated protein degradation (Hubbard et al., 1997). *Sel-10* also interacts genetically with the *C.elegans* presenilin, *Sel-12* (Wu et al., 1998). Thus, SEL-10 may target both Notch and presenilin.

To elucidate the mechanisms by which SEL-10 regulates Notch/LIN-12 signaling, we analyzed a human homologue of *C. elegans* SEL-10 in order to carry out molecular and biochemical studies in mammalian cells. We demonstrate that human SEL-10 physically interacts with mammalian Notch proteins in a domain-specific manner. We also show that Notch proteins are phosphorylated and the interaction between SEL-10 and Notch proteins is very likely phosphorylation-dependent. In addition, Notch proteins are ubiquitinated and degraded by the 26S proteosome in the cell, and through an *in vitro* ubiquitination assay, we show that SEL-10 can mediate Notch protein ubiquitination. The role of SEL-10 in Notch ubiquitination and degradation is also supported by the fact that a SEL-10 deletion mutant containing only

the WD40 repeats can stabilize Notch proteins, presumably by competing with wild-type SEL-10 for binding to Notch.

RESULTS

Human SEL-10, an F-box/WD40 protein that negatively regulates Notch signaling

Figure 1 presents a schematic of the identified domains of the human SEL-10 protein. The full coding sequence for human *SEL-10* is predicted to encode a 540 amino acid protein (GenBank Assession # to be obtained from GenBank). Like its homologue in *C..elegans*, human SEL-10 contains a N-terminal F-box domain followed by seven WD repeats. The predicted protein sequences of *C.elegans* SEL-10 and human SEL-10 show 47.6% identity and approximately 57% similarity. Higher conservation is exhibited when comparing the WD40 repeat domains (60% identity) then in the N-terminus and F-box domain (30% and 35% identity, respectively).

To explore the functional activity and binding potential of human SEL-10 we added the coding sequence for 6 consecutive myc epitope tags (6Xmyc) to the N-terminus of SEL-10. We also generated two variants of SEL-10: SEL-10Fmyc encodes the N-terminus through the F-box and terminates just prior to the first WD40 repeat and SEL-10 WDmyc encodes the WD40 repeats but not the F-box and sequences upstream of the F-box. Based upon analogies to other mammalian member of the F-box/WD40 repeat family proteins it is predicted that the F-box may mediate association with the ubiquitination machinery and the WD40 repeats may provide a binding domain for substrates. Engineered versions of other F-box/WD40 protiens that encode only the WD40 repeats, analogous to SEL-10WDmyc (Fig. 1B), have been shown to function as

dominant-negative proteins, β-TRCP for example (Kitagawa et al., 1999; Latres et al., 1999).

SEL-10 binds to Notch through the WD40 domain and SKP1 through the F-box

To investigate whether human SEL-10 is involved in Notch protein ubiquitination and degradation, we first studied the physical interaction between human SEL-10 and mouse Notch4 proteins using co-immunoprecipitation assays. For binding studies a variant of the Notch4 protein, originally referred to as int-3, is used. We will refer to this variant as Notch4(int-3) and it contains the entire intracellular domain of mouse Notch4, the transmembrane domain and a short extracellular sequence. Notch4(int-3) is known to function as a gain-of-function mutation of Notch4 (Jhappan et al., 1992; Uyttendaele et al., 2000; Uyttendaele et al., 1998).

Binding assays were conducted after co-expression of the myc-tagged variants of human SEL-10 and a HA-tagged Notch4(int-3) (Uyttendaele et al., 1998). Bosc23 cells, derivative of human HEK 293 cells, were used for transient transfections with expression constructs. Cell extracts were prepared and used for immunoprecipitations with either anti-HA or anti-myc antibodies. Immunoprecipitations followed by Western blotting with the same antibodies demonstrate that Notch4(int-3)HA (Figure 2A, bottom panel) and all three SEL-10 variants (Figure 2A, second panel from top) were expressed at comparable levels and immunoprecipitated properly. By probing the anti-myc immunoprecipitates with anti-HA antibody, we demonstrate that Notch4(int-3)HA can be detected in the immune complex of either full length human SEL-10 (Figure 2A, top panel, Lane 6) or the WD40 repeats (Figure 2A, top panel, Lane 8), but not that of the F-box domain (Figure 2A, top panel, Lane 7). We confirmed the

interaction between Notch4(int-3) and SEL-10 proteins by immunoprecipitating Notch4(int-3)HA first with anti-HA, and then probing for myc-tagged SEL-10 proteins. As shown in Figure 2A (third panel from top), full length human SEL-10 (Lane 6) but not the F-box domain (Lane 7) can complex with Notch4(int-3)HA. SEL-10WDmyc co-migrates with the heavy chain of immunoglobulin and therefore, can not be visualized in Figure 2A, third panel from top, Lane 8. In conclusion, human SEL-10 physically associates with mouse Notch4(int-3) through the WD40 repeat region, whereas the F-box domain is not required for this interaction.

Based on previous studies of CDC4 family proteins, we predict that the F-box domain of SEL-10 should interact with other components of the ubiquitination machinery, such as SKP1. We tested this possibility by co-immunoprecipitation assays using HA-tagged full length human SKP1 and myc-tagged SEL-10 proteins. As shown in Figure 2B (top panel, lanes 6 ,7 and 8), SKP1HA interacts with both full-length human SEL-10 and the F-box domain, but not the WD40 repeats. Consistent with this observation, we were able to detect full-length SEL-10 and the F-box domain in the immunoprecipitates of SKP1 (Figure 2B, third panel from top, lanes 6 and 7). The interaction of SKP1 with the F-box domain of human SEL-10 indicates that SEL-10, like other CDC4 family proteins, is part of an E3 ubiquitin ligase that mediates the ubiquitination and degradation of target proteins.

The Notch4 C-terminal domain is critical for binding to human SEL-10.

To map the domain of Notch4(int-3) that is required for the physical interaction between Notch4 and SEL-10, we tested a series of Notch4(int-3) deletion variants (schematized in Fig. 3A) for their ability to complex with full length human SEL-10.

Co-expression of myc tagged SEL-10 and HA-tagged Notch4(int-3) variants was followed by immunoprecipitations of SEL-10 with myc antibody, then the immunoprecipitates were probed with anti-HA. This co-immunoprecipitation assay shows that Notch4(int-3), Notch4(int3)ΔNT, and Notch4(int-3)ΔCDC proteins interact with SEL-10 (Figure 3B, top panel, lanes 8, 9, 12). When the co-immunoprecipitation was conducted in a complementary fashion by immuno-precipitating with anti-HA first for Notch4 proteins and then probing with anti-myc for SEL-10, we could detect SEL-10 in the immune complex of Notch4(int-3) or Notch4(int-3)ΔNT (Figure 3B, third panel from top, Lane 8, 9). Taken together, these results suggest that the C-terminal domain of Notch4(int-3), distal to the CDC10/Ankyrin repeats, is responsible for interacting with SEL-10. All three of the Notch4(int-3) variants that interact with SEL-10 contain the C-terminal domain and removal of this domain abolishes the interaction between Notch4(int-3) and SEL-10.

To address whether the C-terminal domain of Notch4(int-3) alone is sufficient for binding to SEL-10, we analyzed the interaction between full length human SEL-10 and a Notch4(int-3) fragment containing only the C-terminal domain, Notch4(int-3)C, in co-immunoprecipitation assays. Notch4(int-3)C could be detected after expression in Bosc23 cells (Fig. 3C, middle panel, lanes 2, 4, 6, 8). However, when co-immunoprecipitations were carried out under standard conditions, as described above, we failed to detect a complex between Notch4(int-3)CHA and SEL-10myc. In order to prevent ubuqitin-mediated turnover, we treated cells with a specific proteasome inhibitor, lactacystin, before harvest. After lactacytin treatment we detected Notch4(int-3CHA proteins in the immunoprecipitates of SEL-10myc (Figure 3C, top panel, lane 8). Thus the interaction between Notch4(int-3)C and SEL-10 is likely transient and unstable and inhibition of proteasome activities stabilizes the complex.

SEL-10 binds to phosphorylated forms of Notch4(int-3) proteins.

Upon Western blot analysis of Notch4(int-3) variants (Fig. 3B, bottom panel, lanes 9, 12) we noted that some of the Notch4(int-3) proteins migrate as multiple bands. We speculated that this may represent Notch proteins modified by phosphorylation. To address this possibility we determined if the pattern of migration could be altered after phosphatase treatment of immune-complexed Notch4(int-3) proteins.

We chose to focus on the Notch4(int-3)ΔNT variant. SEL-10myc and Notch4(int-3)ΔNTHA were co-expressed in Bosc23 cells. When immune-complexes containing Notch4(int-3)ΔNTHA was probed by Western blotting with anti-HA antibodies three bands ranging from 48 to 51 kDa were detected (Figure 4, lane 1, arrows). After treatment with calf intestinal phosphatase (CIP), the top two bands were significantly diminished indicating that they had become de-phosphorylated (Fig. 4, lane 2). We next determined which forms of Notch4(int-3)ΔNT would associate with SEL-10. The same cell lysate was first immunoprecipitated with anti-myc antibody to bring down SEL-10myc and then probed with anti-HA. The Notch4(int-3Δ)NHA proteins that associated with SEL-10 was predominantly from the slowest migrating form (Fig. 4, lane 3, top arrow). This immune-complex was treated with CIP and the slower migrating form was diminished and the faster migrating forms increased. (Fig. 4, lane 4). This observation suggests that human SEL-10 preferentially binds to the phosphorylated forms of Notch4(int-3) and is consistent with the predicted behavior of CDC4-like proteins, which are thought to bind to phosphorylated target proteins. We noted that Notch4 variants that contained the C-terminus typically migrated as several species (N4(int-3)ΔNT, N4(int-3)ΔCDC and N4(int-3)C). Several other these Notch4(int-3) variants were also tested in this assay and similarly, the slower migrating forms of

these variants were diminished after phosphatase treatment. Taken together with the results shown in Figure 3B and C, we conclude that the C-terminus of Notch4(int-3) is both a site of phosphorylation and the domain required for Notch4/SEL-10 interaction.

Notch proteins can be stabilized by proteasome inhibitors and a dominant-negative form of human SEL-10

To study whether Notch proteins are degraded by the ubiquitination pathway cells expressing Notch proteins were treated with specific proteasome inhibitors. After treatment, Western blot analysis was used to measure the changes in the steady state levels of these proteins. A protein encoding the C-terminal tail of Notch4(int-3) is normally expressed poorly in transfected Bosc23 cells (Fig. 5B, 0 hr. time point) but the levels of this protein is significantly increased after lactacystin treatment (Fig. 5B), indicating turnover by the proteasome. Interestingly, Notch4(int-3) is expressed well in transfected Bosc23 cells (Fig. 2A, bottom panel) and its expression is not significantly increased after lactacystin treatment (data not shown). Thus the Notch4(int-3) protein is not efficiently processed by the proteosome whereas a fragment containing the C-terminal tail of Notch4(int-3) is. A membrane-tethered form of murine Notch1 protein, myc-N1ΔE (Schroeter et al., 1998), is stabilized by lactacystin treatment (Fig. 5B). A protein containing the entire intracellular domain of murine Notch1, N1ICHAHis, is stabilized by treatment with another specific proteasome inhibitor, MG132 (Fig. 5C). Taken together, steady state levels of intracellular Notch proteins are increased upon treatment with proteasome inhibitors indicating they are targeted for degradation via the ubiquitination pathway.

To determine whether the increased steady state levels of Notch proteins are a result of the increased half-life of the proteins we carried out pulse-chase analysis to

assess the half-life of the Notch4(int-3)C protein, the C-terminus of Notch4(int-3). Figure 5D shows that in the absence of lactacystin, more than half of the Notch4(int-3)C protein is turned over after approximately 1.5 hours of chase. On the contrary, after treatment with 10μM lactacystin, the level of Notch4(int-3)C remained relatively steady through the chase period of 2.5 hours. Based on this result, the increase of Notch4(int-3)C levels after lactacystin treatment shown in Figure 5A is likely the result of decreased turnover of the protein. This prolonged half-life upon treatment by a proteasome inhibitor indicates that Notch proteins are degraded via the ubiquitination pathway.

To determine if endogenous SEL-10 is required to target Notch proteins for turnover we engineered a form of SEL-10 predicted to interfere with endogenous SEL-10 function (See Figure 1B, SEL-10WDmyc). CDC4 family proteins function in a modular fashion, with WD40 repeat region binding to the target protein and F-box region binding to SKP1. Studies of other F-box/WD40 proteins have shown that expression of the WD40 repeat domain alone can function as a dominant-negative, interfering with endogenous protein function (Latres et al., 1999). We tested the ability of SEL-10WDmyc, which only encodes the WD40 repeats and not the F-box, to interfere with endogenous SEL-10 function by co-expressing SEL-10WDmyc with either Notch4(int-3)C or N1ICHA in Bosc23 cells. Two days after transfection, cells were harvested and the steady state levels of Notch proteins were examined by Western blot analysis. Expression of SEL-10WD resulted in increased steady state levels of both Notch4(int-3)C (Fig. 6A) and N1IC (Fig. 6B). This increase was apparent even when equivalent levels of each expression plasmid was used. Further increases in protein levels occurred in a dosage dependent manner as more SEL-10WD plasmid was used. The increased expression of Notch4(int-3)C after expression of SEL-10WD was reflected in increased half-life of the protein (Fig. 6C). The half-life of Notch4(int-3)C was

approximately doubled after expression of SEL-10WD (Fig. 6C). Thus WD40 repeat region of SEL-10 functions as a dominant negative form of SEL-10 and expression of this form results in decreased turnover of Notch proteins.

SEL-10 mediates Notch protein ubiquitination *in vitro*

To address whether SEL-10 functions as part of an SCF ubiquitin ligase that targets Notch4(int-3) proteins for ubiquitin-dependent degradation we first tested if SEL-10myc and SEL-10WDmyc assembled into SCF complexes. Both proteins were co-expressed in insect cells with hCUL1, hSKP1, and hHRT1, and SEL-10 protein complexes were retrieved by immunoprecipitation with anti-myc antibody beads. Whereas full length SEL-10 efficiently co-precipitated hCUL1, hSKP1, and hHRT1, the WD40 repeat domain by itself was unable to recruit the other SCF subunits (Fig. 7A), thus providing an explanation for the observed dominant-negative effect of SEL-10WDmyc in transfected cells. We next tested if Notch4(int-3) was ubiquitinated by recombinant SCF^{SEL-10}. As shown in Fig. 7B, high molecular weight (HMW) forms of Notch4(int-3)HA, Notch4(int-3)CHAHis, and N1ICHAHis proteins were generated in the presence of SEL-10myc immunoprecipitates that contained all subunits of SCF, but not by SEL-10WDmyc immunoprecipitates. Formation of the HMW forms of Notch4 proteins was dependent on the presence of ubiquitin, confirming that Notch4 ubiquitination was reconstituted in the presence of SCF^{SEL-10}. We then looked in more detail at N1ICHAHis ubiquitination in the presence or absence of Ub and its chain-terminating derivative methyl Ub. In the presence of Ub and SCF^{SEL-10}, SCF-bound N1ICHAHis was completely converted into HMW conjugates. Substituting ubiquitin with methyl ubiquitin dramatically reduced the apparent size of the ubiquitinated N1ICHAHis, whereas ommission of ubiquitin from the reaction completely abolished

formation of HMW conjugates. These results confirm that the HMW forms observed were indeed ubiquitinated forms of N1ICHAHis. As expected, immunoprecipitates that contained full length SEL-10,, but not those that contained SEL-10WD, sustained N1ICHAHis ubiquitination *in vitro*.

DISCUSSION

Although the mechanisms of Notch/LIN-12 signal transmission are being defined through genetic and biochemical analysis, little is known about the mechanisms involved in down-regulating the Notch signal. Sel-10 was originally identified in *C.elegans* as a negative regulator of Lin-12 activity (Hubbard et al., 1997). The fact that the SEL-10 protein is related to the CDC4 family of F-box/WD40 repeat proteins suggested that SEL-10 down-regulates Notch/LIN-12 signaling by targeting these proteins for ubiquitin-mediated protein turnover (Hubbard et al., 1997). F-box/WD40 proteins predict bind their target proteins in a phosphorylation-dependent fashion. We demonstrate here that the WD40 repeats of SEL-10 bind to the C-terminal domain of the Notch4 protein, a domain where Notch4 is phosphorylated. SEL-10 preferentially binds to the phosphorylated forms of Notch4, suggesting the interaction is phosphorylation-dependent. We also found that several forms of Notch proteins are very labile and this is a result of rapid turnover via the ubiquitination-proteosome pathway. Interfering with endogenous SEL-10 activity by over-expression of the WD40 repeat region of SEL-10 blocks Notch protein turnover, indicating SEL-10 is directly involved in mediating Notch ubiquitination and degradation. Finally, in vitro ubiquitination of Notch proteins occurs only in the presence of SEL-10. Thus, SEL-10 functions as a component of a SCF complex to target Notch proteins for ubiquitination and degradation.

Other reports have also established that Notch proteins are likely turned over by ubiquitination. For example, it has been reported that the steady state level of Notch1 intracellular domain can be elevated by lactacystin, a proteasome inhibitor (Schroeter et al., 1998). In addition, *Notchless*, a novel *Drosophila* gene identified as a modulator of *Notch* activity encodes a WD40 repeat containing protein which binds to the intracellular domain of Notch (Royet et al., 1998). However, the function of *Notchless* is

not clear because both loss-of-function mutations and over-expression of the gene lead to increased Notch activity. This, once again, suggests that the regulation of Notch pathway is very complex. A recent report defines Notch proteins as targets for ubiquitination and provides biochemical evidence that the Itch protein may participate in mediating Notch ubiquitination. However, in this study it was not established that Itch is responsible or participates in the ubiquitination of Notch in vivo or that Notch ubiquitination, stability or activity is altered in the Itchy mouse which carries an *Itch* mutation.

An interesting observation is that Notch4(int-3) shows strong and specific interaction with SEL-10 but does not seem to be readily degraded by the ubiquitination pathway, in contrast to Notch1IC. Over-expression of the WD40 repeat region or treatment by lactacystin failed to increase the steady state levels of Notch4(int-3) (data not shown). Pulse-chase analysis indicate that Notch4(int-3) has a much longer half-life in cells than does Notch4(int-3)C, the C-terminal domain of Notch4(int-3) (data not shown). However, Notch4(int-3) still seems to be ubiquitinated in the cell because a Western blot of Notch4(int-3) often displays a very high molecular weight smear in addition to the main Notch4(int-3) signal at the predicted molecular weight (unpublished observations). This smear can be seen even without proteasome inhibitor treatment and is very typical of proteins that are ubiquitinated. One possible explanation to these observations is that the transmembrane domain and the extracellular sequence in Notch4(int-3) can function to prevent the protein from being degraded by proteasome even after ubiquitination. The resultant increased stability of Notch4(int-3) may also contribute to the potent oncogenic activity of this variant of Notch4 which was originally defined as a mammary oncogene (Jhappan et al., 1992; Robbins et al., 1992). In contrast, our studies show that Notch4(int-3)C and N1IC, both

lacking a transmembrane domain and extracellular sequence, can be readily stabilized by proteasome inhibitors or over-expression of the WD40 repeat region of SEL-10. This issue can be further addressed by biochemical studies using an Notch4(int-3) fragment containing only the intracellular domain or possibly chimeric proteins between Notch4(int-3) and Notch1IC.

We conclude that the C-terminal domain of Notch4 distal to the CDC10/Ankyrin repeats is a negative regulatory domain because it is responsible for interaction with SEL-10. This is consistent with the fact that the C-terminal domain contains a PEST sequence, which is characteristic to many short-lived proteins and is thought to be a target for ubiquitination (Rechsteiner and Rogers, 1996). It has also been observed that a C-terminal deletion can activate GLP-1, a *C. elegans* Notch protein (Mango et al., 1991). The C-terminal domain of Notch proteins is also where some other regulatory proteins bind. For example, *Drosophila* protein Dishevelled has been reported to bind to this region and may thus mediate the interaction between the Wingless and Notch signaling pathways (Axelrod et al., 1996).

Ubiquitin-mediated protein degradation is a highly regulated and highly selective process and is used to downregulate several signaling pathways (Aberle et al., 1997; Chen et al., 1995). We report that Notch signaling also utilizes ubiquitin-mediated protein turnover to down-regulate the Notch/LIN-12 signal. This is evident both in *C.elegans* (Hubbard et al., 1997) and for Notch signaling in mammalian cells (Fig. 1C and D). Understanding of how Notch proteins are regulated by this pathway may provide new approaches to control Notch activity. For example, constitutive activation of Notch can lead to tumorigenesis, SEL-10 activity could potentially be used to reverse Notch activity in these circumstances.

CDC4 family proteins can bind to multiple target proteins (Margottin et al., 1998; Winston et al., 1999b; Yaron et al., 1998). Besides Notch, SEL-10 also interacts with presenilin proteins in co-immunoprecipitation assays and it may be responsible for targeting presenilin proteins for ubiquitination-mediated protein degradation (Wu et al., 1998). Strong evidence has been presented supporting the fact that presenilin is required for the activation of Notch, probably because it is involved in the proteolytic cleavage and nuclear access of Notch intracellular domain (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). But it is not clear whether SEL-10 has any effects on the presenilin/Notch interaction.

Our results predict that Notch levels and activity may be controlled by kinase(s) that phosphorylate the C-terminus of Notch proteins. This phosphorylation would mediate SEL-10 binding and thus targeting to the proteasome. Little is known about kinases that phosphorylate and regulate Notch but one would predict that the kinase the phosphorylates the C-terminus has a negative regulatory function in Notch signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Media

Bosc23 cells (Pear et al., 1993) were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum and penicillin-streptomycin. Sf9 insect cells were maintained in Gibco BRL SF900II medium. DH10Bac Bacteria strain was purchased from Gibco BRL.

Plasmids and Vectors

The following plasmids were constructed in pQNCIIX (Julius et al., 2000), a retroviral vector that drives gene expression under the control of a CMV promoter.

(B) pQNClacZ contains the bacterial lacZ gene.

(C) pQNCint-3CHAHis expresses Notch4(int-3)CHAHis, a C-terminal fragment of mouse Notch4 protein (amino acids 1789-1964) with a HA and 6 His tags at the C-terminus.

(D) pQNCNotch1ICHAHis expresses rat Notch1 intracellular domain (amino acids 1747-2531) with a HA and 6 His tags at its C-terminus.

The following plasmids were constructed in pLNCX, a retroviral vector that drives gene expression under the control of a CMV promoter. These plasmids express different regions of the Notch4(int-3) protein, and have been described previously (Miller and Rosman, 1989).

(E) pLNCint-3HA contains cDNA corresponding to the *Notch4* region expressed in the int-3 insertion, beginning at amino acid 1411; the Notch4(int-3) protein includes the

entire intracellular domain of Notch4 and additional sequences. The entire protein is HA-tagged at the C-terminus.

(F) pLNCint-3ΔNTHA expresses ΔNTHA, an Notch4(int-3) protein lacking the region upstream of the CDC10/Ankyrin repeats.

(G)pLNCint-3ΔCTHA expresses ΔCTHA, an Notch4(int-3) protein lacking the region distal to the CDC10/Ankyrin repeats.

(H)pLNCint-3ΔNTΔCTHA expresses the CDC10/Ankyrin repeat region of Notch4(int-3).

(I) pLNCint-3ΔCDCHA expresses an Notch4(int-3) protein lacking the CDC10/Ankyrin repeats.

All the above Notch4(int-3) proteins have an in-frame HA tag at the C-terminus.

The following plasmids were constructed in pCS2-MT6 (Roth et al., 1991), a vector that drives gene expression under the control of a CMV promoter. There are six myc tags upstream of the polyclonal sites.

(J) pCS2hSEL-10myc expresses full-length human SEL-10 protein with 6 myc epitope tags at the N-terminus.

(K) pCS2hSEL-10WDmyc expresses the WD40 repeat region (amino acids 184-540) of human SEL-10 with 6 myc epitope tags at the N-terminus.

(L) pCS2hSEL-10Fmyc expresses the F-box region (amino acids 1-207) of human SEL-10 with 6 myc epitope tags at the N-terminus.

(M) pCS2HA-HsSKP1 expresses HA-tagged full length human SKP1 (Lyapina et al., 1998).

(N)pCS2mN$^{\Delta E}$ expresses a 6Xmyc tagged murine Notch1 with deletion of the extracellular domain (De Strooper et al., 1999). It is a gift from Dr. Rapheal Kopan.

The following plasmids were constructed in pFASTBAC (from Gibco BRL), a shuttle vector for making baculoviruses over-expressing proteins in insect cells. For details, see Gibco BRL catalog.

(O) pFastBacInt-3HA contains the entire *Notch4(int-3)* sequence fused to a C-terminal sequence encoding the HA epitope tag.

(P) pFastBacInt-3CHAHis encodes the C-terminal region (amino acids 1789-1964 of mouse Notch4) of Notch4(int-3) fused at its C-terminus to a HA and 6 His tags.

(Q) pFastBacN1ICHAHis encodes the intracellular domain (amino acids 1747-2531) of rat Notch1 fused at its C-terminus to a HA and 6 His tags.

(R) pFastBachSEL-10myc expresses full-length human SEL-10 with 6 N-terminal myc epitope tags.

(S) pFastBachSEL-10WDmyc expresses the WD40 repeat region (amino acids 184-540) of human SEL-10 with 6 N-terminal myc tags.

Transfection, Immunoprecipitation, and Western blot analysis

For transient transfection, a confluent plate of Bosc23 cells was split 1:3 the day prior to the transfection. For one 60mm plate of cells, 4μg of each plasmid DNA was transfected using the calcium phosphate precipitation method. The total amount of DNA was kept constant by supplementation with lacZ-containing plasmids.

Two days after transfection, cells were harvested and lysed in TENT buffer (50mM Tris·Cl (pH8.0), 2mM EDTA, 150mM NaCl, 1% Triton-X 100) containing protease inhibitors (2μg/ml aprotinin, 2μg/ml leupeptin, 2μg/ml pepstatin, 0.5mM PMSF). Lysates were clarified by centrifugation at 10,000g for 10 minutes and protein content was determined using the BioRad Protein Assay Kit. Equal amount of extract from each sample was pre-cleared with Sepharose CL-4B beads, incubated with

antibodies (50μl of 12CA5 anti-HA supernatant, 200μl of 9E10 anti-myc supernatant, or 2μl of anti-FLAG antibody) for 2 hours at 4°C, then incubated with 40μl of 50% slurry of protein A-Sepharose for 1 hour at 4°C. The protein A-Sepharose beads were then washed with TENT buffer three times by vortexing for 5 minutes each time. The beads were boiled in 30μl 1X protein loading buffer, and then electrophoresed on a SDS-polyacrylamide gel and transferred onto PVDF membrane.

A Western blot was first blocked overnight at 4°C with TBST (10mM Tris, pH 8.0, 150mM NaCl, 0.2% Tween 20) containing 1% bovine serum albumin (TBST-BSA). The blot was then incubated with 1° antibody diluted (1:50 for 12CA5; 1:10 for 9E10; 1:2000 for anti-FLAG) in TBST-BSA for 1 hour, washed three times for 5 minutes each with TBST, and incubated with 2° antibody in TBST-BSA for 1 hour. After three washes, the signal was visualized by chemiluminescence (Amersham, ECL).

12CA5 anti-HA antibody was obtained from Berkeley Antibody Co., Richmond, CA. 9E10 anti-myc antibody was prepared from culture supernatants of the 9E10 hybridoma (Kolodziej and Young, 1991). Anti-FLAG antibody was purchased from Sigma.

Dephosphorylation of proteins with calf intestinal phosphatase (CIP)

Immunoprecipitation was first carried out to bring down the protein to be treated with CIP. At the end of the immunoprecipitation, the protein A-Sepharose beads were thoroughly washed with TENT, and the solution was completely removed by aspiration. The beads were then suspended in 30μl of 1X CIP buffer containing 2μl of CIP (New England Biolab), and incubated at 37°C for 2 hours. 10μl of 4X protein gel loading buffer was then added to the reaction. The sample was boiled, loaded onto SDS-PAGE gel, and then subjected to Western blot analysis.

Pulse-Chase Labeling Assay

Bosc23 cells were transfected with plasmid DNA as described above. Two days after transfection, the cells were first washed and incubated in DMEM medium lacking methionine (Met) and cysteine (Cys) for half an hour to deplete Met and Cys. Cells were then incubated for half an hour in DMEM labeling medium containing ^{35}S-labeled Met and Cys at 0.5mCi/ml. The labeling medium was then replaced with regular DMEM medium. Cells were harvested every half an hour during the chase period for up to 2.5 hours. 10μM of Lactacystin was added to both pulse labeling and chase media when needed.

The harvested cells were then lysed and immunoprecipitated as described above. The precipitates were separated on SDS-PAGE gel, and the gel then fixed for 30 minutes in isopropanol:water:acetic acid (25:65:10), stained for 30 minutes with Amplify™ (Amersham), vacuum-dried and exposed to X-ray film to visualized the signal.

Generation of Baculovirues and in vitro Ubiquitination Assay

Baculoviruses used in the in vitro ubiquitination assays were generated with the Gibco BRL FastBac system following the manufacurer's protocols. Hi5 insect cell lysates were prepared 48 hr post-infection from cells co-infected with baculoviruses that expressed SEL-10myc or SEL-10WDmyc, plus hCUL1, hSKP1, and hHRT1. Cell lysis was achieved by incubating cells in buffer containing 20mM Hepes, pH7.4, 150 mM NaCl, 50m M NaF, 60 mM β-glycerophosphate, 0.3% Triton, 100 μM LLnL, and 1X protease inhibitor cocktail (Sigma). Crude Hi5 cell lysates (500 μg) were incubated with 10 μl of anti-Myc beads for 2 hr at 4°C to allow binding of myc-tagged SEL-10 subunits. Beads were washed three times with lysis buffer and incubated with 500 μg of crude

lysates prepared from Hi5 cells infected with baculoviruses that expressed either Notch4(int-3)HA, Notch4(int-3)CHAHis6, or N1ICHAHis6 to allow substrate binding to SCF. Beads were washed three times with lysis buffer, two times with 20mM Hepes, pH7.4, 100 mM potassium acetate, 1 mM DTT, and incubated with the following ubiquitination reaction components: 50 ng ^{His6}yUBA1, 500 ng hCDC34, 1 µl of 10X ATP-regenerating system, 1 µl of 10X reaction buffer (Feldman et al., 1997) and 5 µg of either ubiquitin or, methyl ubiquitin (Boston Biochem). Ubiquitination reactions were carried out for 60 min at 30°C and terminated by addition of Laemmli sample buffer. The samples were fractionated by SDS-PAGE and Notch proteins were visualized by Western blotting with anti-HA antibodies.

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FIGURE LEGENDS

Figure 1. Human SEL-10 and Its Effects on Notch signaling

(A) Schematic comparison of the structure and similarity of C. elegans and human SEL-10 proteins. Percentage numbers indicate amino acid identities between homologous domains of the two proteins.

(B) Schematic of epitope-tagged human SEL-10 and engineered variants. SEL-10F myc encodes the F-box domain. SEL-10WD40 myc encodes the WD40 repeat domain.

(C)

(D)

Figure 2. Human SEL-10 Interacts with Notch through the WD40 Repeats and SKP1 through the F-box

(A) Physical interactions between human SEL-10 and mouse Notch4. HA tagged Notch4(int-3) protein was co-expressed with the three myc-tagged SEL-10 variants in Bosc23 cells by transient transfection. Immunoprecipitation and Western blotting were performed using either anti-HA or anti-myc antibodies, as indicated, to demonstrate that the proteins are expressed (second panel from the top and bottom panel). Anti-HA antibody was used to probe anti-myc immunoprecipitates to reveal Notch4(int-3)HA complexed to either SEL-10myc or SEL-10WDmyc (top panel). Similarly, anti-myc was used to probe anti-HA immunoprecipitates to reveal SEL-10 associated with Notch4(int-3)HA (third panel from top).

(B) Physical interactions between human SEL-10 and human SKP1. HA tagged SKP1 was co-expressed with the three SEL-10 variants in Bosc23 cells.

Immunoprecipitation followed by Western blotting using anti-HA or anti-myc to show proteins are expressed (second panel from top and bottom panel). Anti-myc immunoprecipitates were probed with anti-HA (top panel) to reveal SKP1HA associated with the SEL-10, and anti-myc was used to detect SEL-10 in the immunoprecipitates of SKP1HA (third panel from top). Arrows indicate SKP1HA which co-migrates with the antibody light chains.

Figure 3. The C-terminal Domain of Notch Mediates the Interaction between Notch and SEL-10

(A) Epitope tagged Notch4(int-3) deletion variants.

(B) Physical interactions between SEL-10 and Notch4 deletion variants. Myc-tagged SEL-10 was co-expressed with HA tagged Notch4(int-3) deletion variants in Bosc23 cells. All the proteins were expressed (second panel from top and bottom panel) except ΔNTΔCTHA (bottom panel, lanes 5 and 11). Anti-HA antibody was used to detect Notch4 in the immunoprecipiates containing SEL-10myc (top panel), and anti-myc was used to detect SEL-10myc in the immuoprecipitates of Notch4 (third panel from top).

(C) A C-terminal fragment of Notch4 complexes with SEL-10 in the presence of proteasome inhibitor. Notch4(int-3)CHAHis, a fragment of Notch4 with only the C-terminal domain was tested for its ability to co-immunoprecipitate with SEL-10. Prior to lysis, cells were either treated with lactacystin or left untreated. The bottom two panels show that both Notch4(int-3)C and SEL-10 are expressed. Notch4(int-3)C is detected by anti-HA in the immune complex of SEL-10myc (top panel). Arrows in the top two panels indicate Notch4(int-3)CHAHis, and arrow in the bottom panel indicates the major species of SEL-10myc.

Figure 4. SEL-10 Binds to Phosphorylated Notch

Notch4(int-3)ΔNTHA and SEL-10myc were co-expressed in Bosc23 cells by transient transfection. Immunoprecipitation was performed with either anti-HA or anti-myc antibodies. Each precipitate was then divided and one sample was treated with CIP (calf intestinal phosphatase) whereas the other was left untreated. The blot was probed with anti-HA to visualize Notch4(int-3)ΔNTHA precipitated either directly by anti-HA or indirectly by anti-myc. Arrows indicate the three species of Notch4(int-3)ΔNTHA. Stars indicate the heavy and light chains of immunoglobulin.

Figure 5. Notch Proteins are Stabilized by Proteasome Inhibitor Treatment

(A) 2μg of a plasmid expressing Notch4(int-3)CHAHis was transiently transfected into Bosc23 cells. The cells were treated with 10μM of lactacystin before harvest. Western blotting using anti-HA antibody was carried out to assess the steady state expression levels of Notch4(int-3)CHAHis (indicated by an arrow).

(B) The above experiment was done using a 6Xmyc tagged Notch1 fragment with a deletion of the extracellular domain, myc N1ΔE. Treatment by 10μM lactacystin was done for up to 8 hours, lactacystin treatment was also carried out at 100μM for 12 hours. Arrow indicates the steady state levels of myc-N1ΔE revealed by Western blotting using anti-myc antibody.

(C) A Notch1 fragment containing the intracellular domain tagged with HA and six His tags was expressed in Bosc23 cells by transient transfection. Cells were treated with proteasome inhibitor, MG132, for 12 hours before harvest. DMSO was used as a negative control. Arrow indicates the Notch1 protein revealed by Western blotting using anti-HA antibody.

(D) Proteasome inhibitor treatment leads to longer half-life of Notch protein. Bosc23 cells were transfected to express Notch4(int-3)CHAHis. Two-days after the transfection, cells were pulse labeled with ^{35}S methionine and cysteine for 30 minutes, and chased with regular medium for up to 2.5 hours. Samples were harvested every half an hour and then immunoprecipitated using anti-HA antibody. The immunoprecipitates were seperated on SDS-PAGE, autoradiographed to reveal the amount of labeled Notch4(int-3)CHAHis. For cells treated with lactacystin, the proteasome inhibitor was added to both pulse labeling and chase media. Arrows indicate the two bands representing Notch4(int-3)CHAHis.

Figure 6. Over-expression of the WD40 Repeats of SEL-10 Leads to Stabilization of Notch Protein

(A) Plasmids expressing Notch4(int-3)CHAHis and SEL-10WDmyc were co-transfected into Bosc23 cells. Amount of plasmids used is indicated. Two days after transfection, cells were harvested for Western blotting. HA tagged Notch proteins are indicated by the arrows

(B) SEL-10WDmyc was co-expressed with N1ICHAHis, a Notch1 fragment containing the intracellular domain. Two days later, the steady state expression levels of N1ICHAHis (indicated by the arrow) were assessed by Western blotting.

(C) Over-expression of the WD40 repeats of SEL-10 results in increased half-life of Notch4(int-3)CHAHis. Pulse-chase labeling experiment was done in the absence or presence of SEL-10WDmyc to determine the half-life of Notch4(int-3)CHAHis. Metabollically labelled Notch4(int-3)CHAHis (indicated by the arrows) was visualized by immunoprecipitation followed by autoradiography.

Figure 7. SCF^{SEL-10} Mediates Notch Ubiquitination in vitro

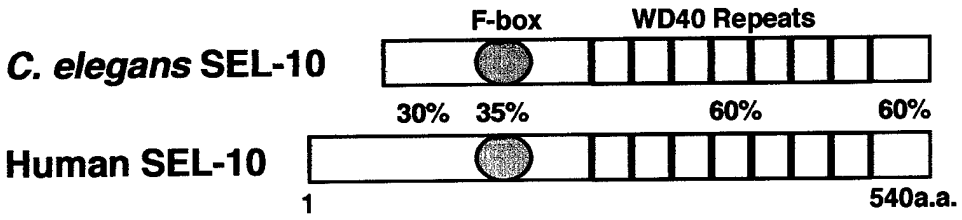
(A) Full length SEL-10 but not the WD40 repeat domain interacts with other SCF components. Hi5 insect cells were co-infected with a cocktail of recombinant baculoviruses that expressed hCUL1, hSKP1, and hHRT1 plus either full length SEL-10myc or SEL-10WDmyc. Crude lysates and anti-Myc immunoprecipitates prepared from infected cells were fractionated by SDS-PAGE and evaluated by Western blotting with anti-Myc, anti-CUL1, anti-SKP1, and anti-HRT1 antibodies, as indicated.

(B) SCF^{SEL-10} mediates ubiquitination of Notch proteins *in vitro*. Bead-bound SCF complexes, prepared as described in (A), were incubated with crude lysates of Hi5 insect cells infected with recombinant baculoviruses that expressed either Notch4(int-3)HA, Notch4(int-3)CHAHis6, or N1ICHAHis6 to allow substrate capture. Beads were washed three times and incubated with ^{His6}yUBA1, hCDC34, and ATP-regenerating

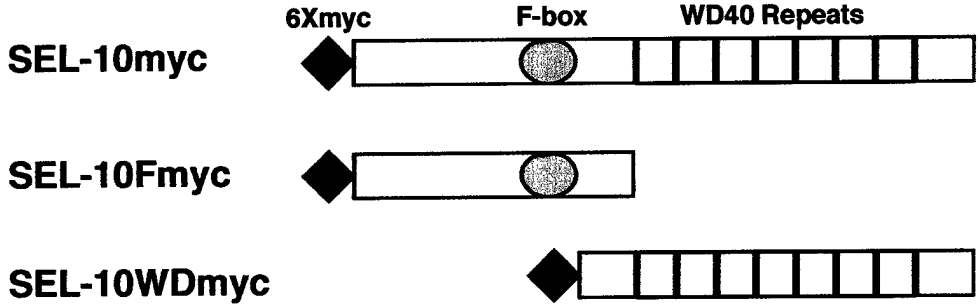
system (Rxn). Ubiquitin was either included or omitted from the reactions as indicated. Following incubation for 60 min at 30°C, the samples were fractionated by SDS-PAGE and Notch4 proteins were visualized by Western blotting with anti-HA antibodies. Arrows designate extensively-ubiquitinated forms of Notch proteins.

(C) N1ICHAHis6 ubiquitination by SCF^{SEL-1} and $SCF^{SEL-10Wdmyc}$. Ubiquitination reactions were performed as described in (B), except that methyl ubiquitin (MeUb) was used where indicated to inhibit multi-ubiquitin chain formation. Arrows designate multi-ubiquitinated forms of Notch proteins.

A



B



C Experiments in progress

D Experiments in progress

Figure 1

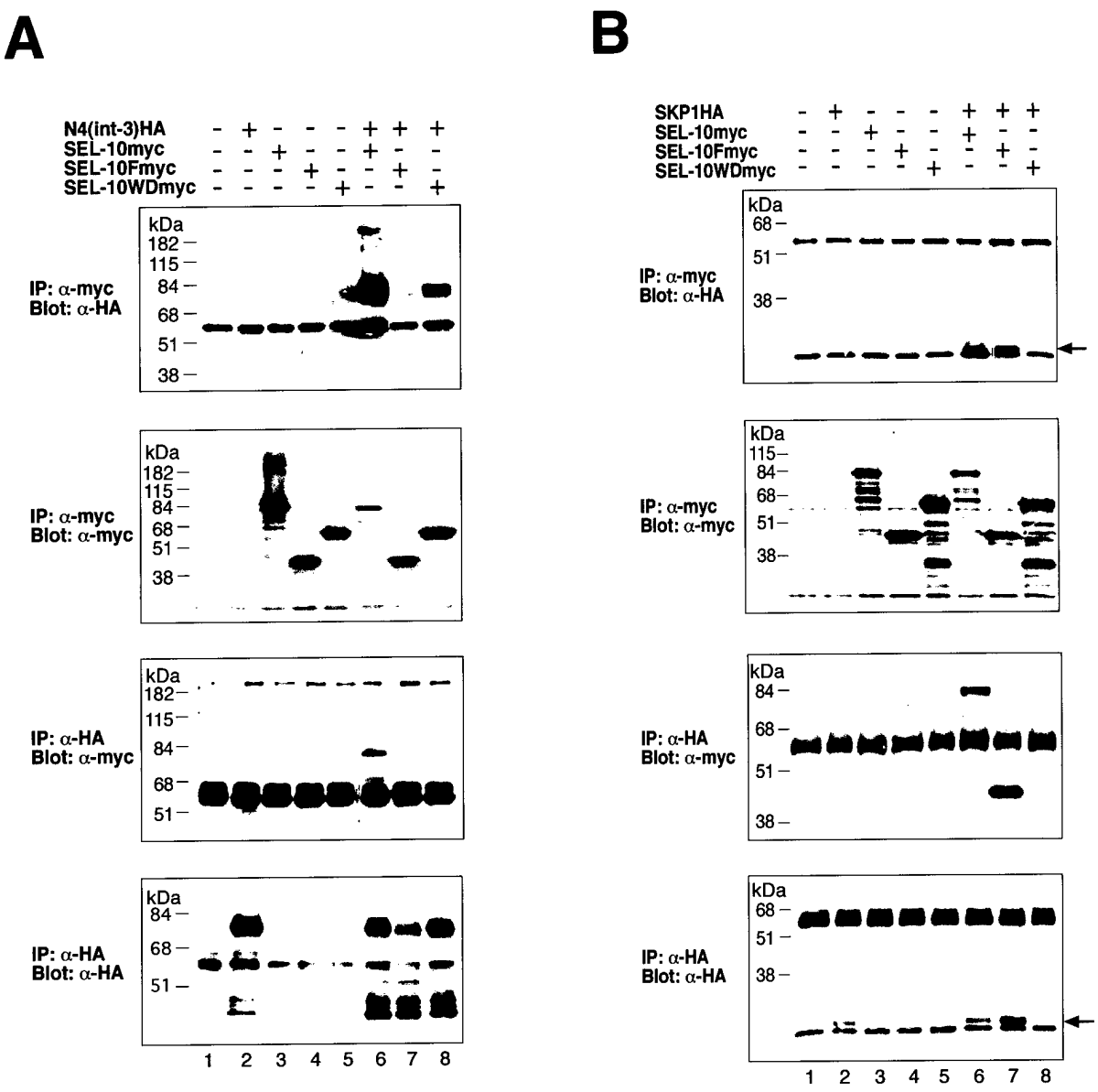

A
N4(int-3)HA - + - - - + + +
SEL-10myc - - + - - + - -
SEL-10Fmyc - - - + - - + -
SEL-10WDmyc - - - - + - - +
IP: α-myc
Blot: α-HA
kDa
182
115
84
68
51
38
IP: α-myc
Blot: α-myc
IP: α-HA
Blot: α-myc
IP: α-HA
Blot: α-HA
1 2 3 4 5 6 7 8
B
SKP1HA - + - - - + + +
SEL-10myc - - + - - + - -
SEL-10Fmyc - - - + - - + -
SEL-10WDmyc - - - - + - - +
IP: α-myc
Blot: α-HA
kDa
68
51
38
IP: α-myc
Blot: α-myc
IP: α-HA
Blot: α-myc
IP: α-HA
Blot: α-HA
1 2 3 4 5 6 7 8

Figure 2

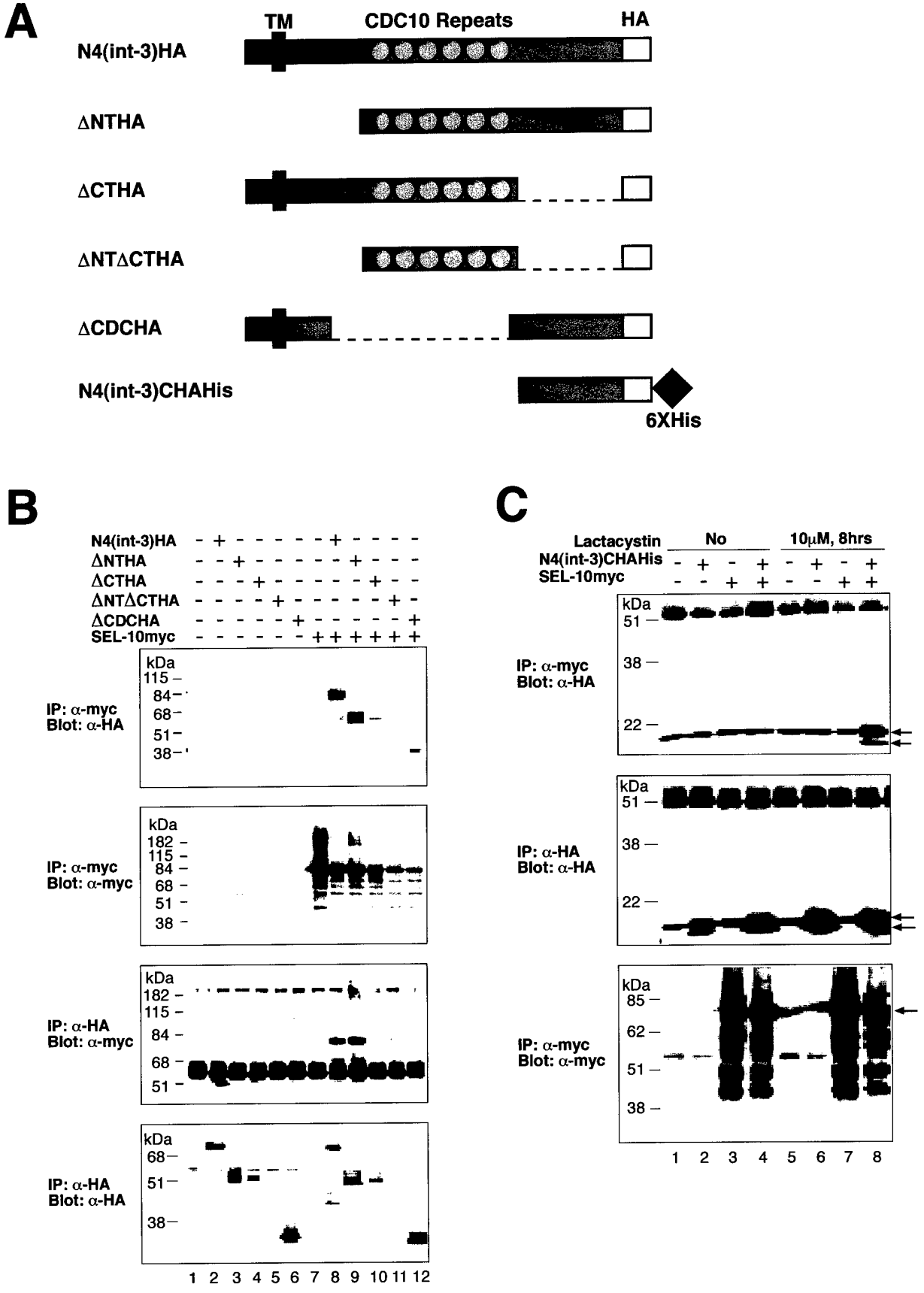

A
TM
CDC10 Repeats
HA
N4(int-3)HA
ΔNTHA
ΔCTHA
ΔNTΔCTHA
ΔCDCHA
N4(int-3)CHAHis
6XHis
B
N4(int-3)HA - + - - - - - + - - - -
ΔNTHA - - + - - - - - + - - -
ΔCTHA - - - + - - - - - + - -
ΔNTΔCTHA - - - - + - - - - - + -
ΔCDCHA - - - - - + - - - - - +
SEL-10myc - - - - - - + + + + + +
IP: α-myc
Blot: α-HA
kDa
115 –
84 –
68 –
51 –
38 –
IP: α-myc
Blot: α-myc
kDa
182 –
115 –
84 –
68 –
51 –
38 –
IP: α-HA
Blot: α-myc
kDa
182 –
115 –
84 –
68 –
51 –
IP: α-HA
Blot: α-HA
kDa
68 –
51 –
38 –
1 2 3 4 5 6 7 8 9 10 11 12
C
Lactacystin
No
10μM, 8hrs
N4(int-3)CHAHis - + - + - + - +
SEL-10myc - - + + - - + +
IP: α-myc
Blot: α-HA
kDa
51 –
38 –
22 –
IP: α-HA
Blot: α-HA
kDa
51 –
38 –
22 –
IP: α-myc
Blot: α-myc
kDa
85 –
62 –
51 –
38 –
1 2 3 4 5 6 7 8

Figure 3

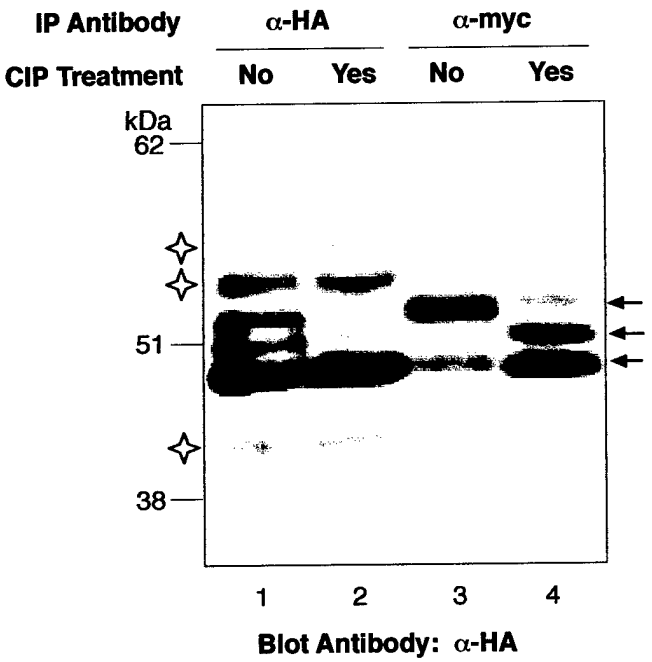

IP Antibody
α-HA
α-myc
CIP Treatment
No
Yes
No
Yes
kDa
62
51
38
1
2
3
4
Blot Antibody: α-HA

Figure 4

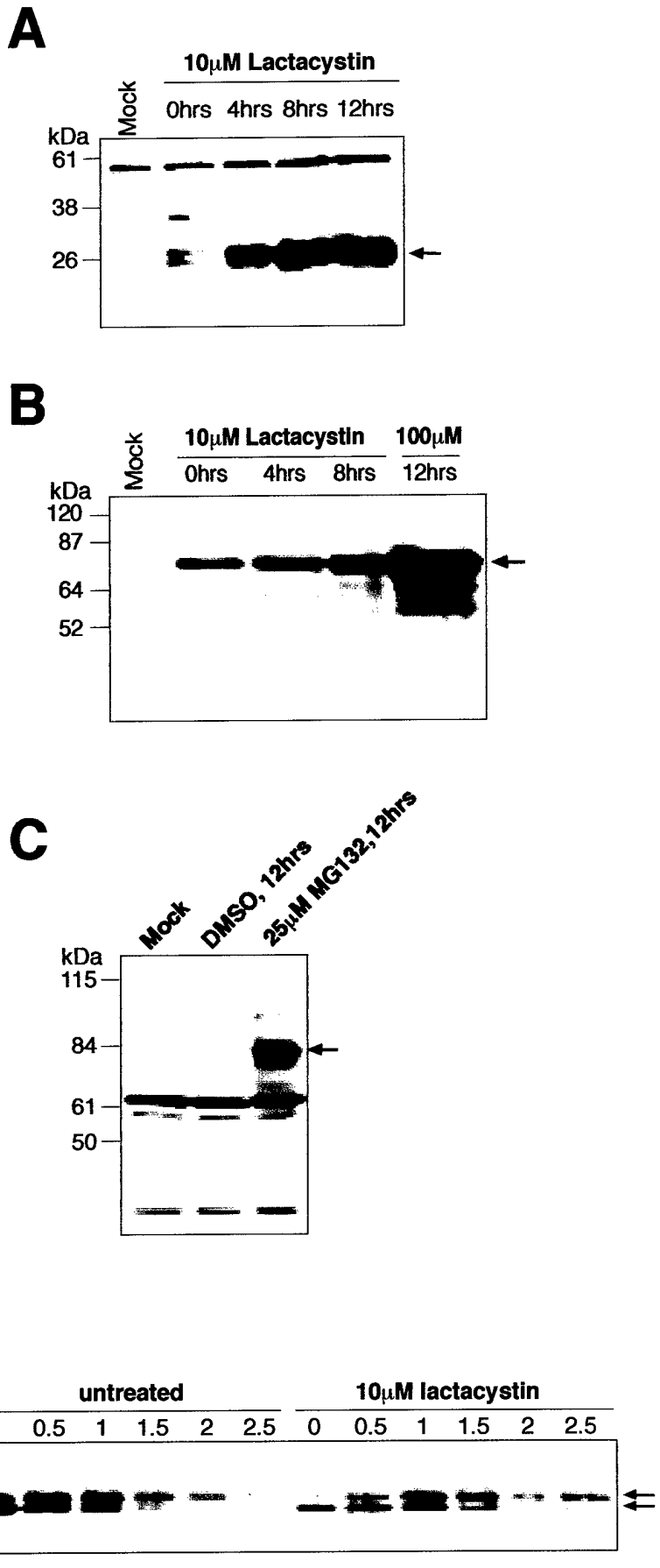

A
10μM Lactacystin
Mock
0hrs 4hrs 8hrs 12hrs
kDa
61
38
26
B
Mock
10μM Lactacystin
100μM
0hrs 4hrs 8hrs 12hrs
kDa
120
87
64
52
C
Mock
DMSO, 12hrs
25μM MG132,12hrs
kDa
115
84
61
50
D
untreated
10μM lactacystin
Chase(hrs) 0 0.5 1 1.5 2 2.5 0 0.5 1 1.5 2 2.5

Figure 5

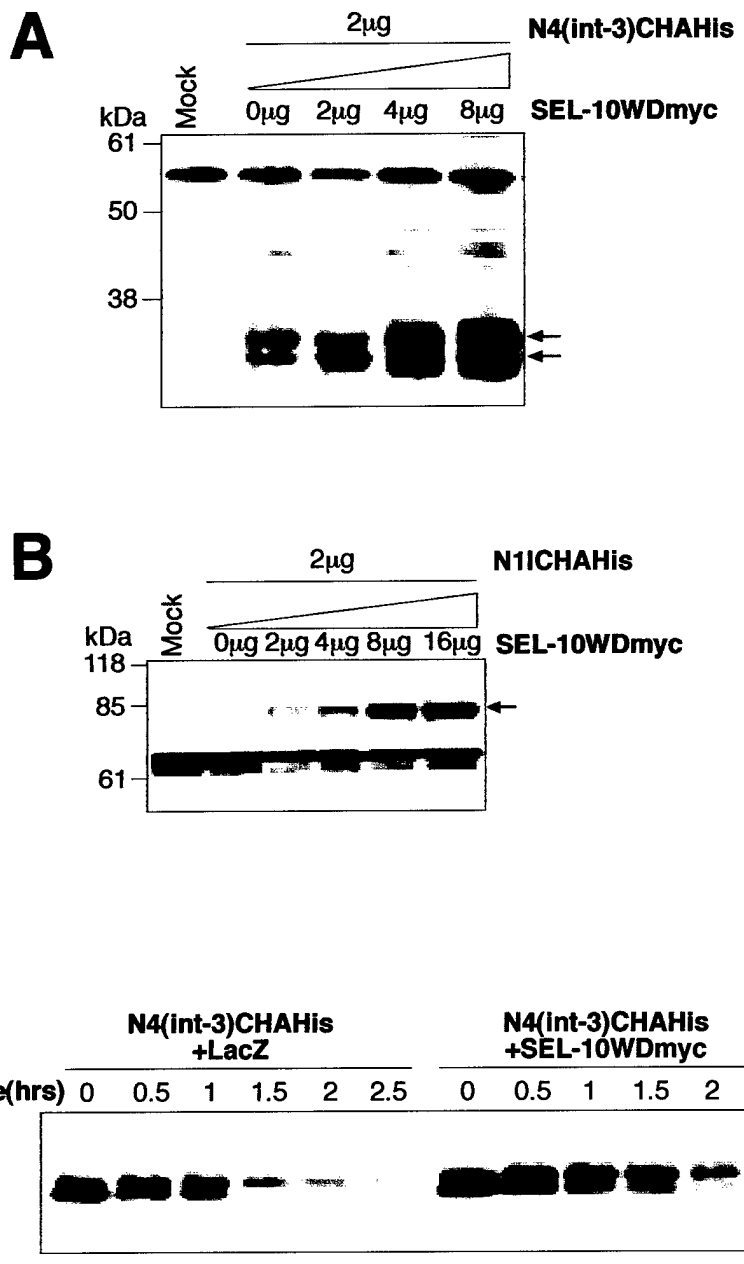

A
2μg
N4(int-3)CHAHis
Mock
0μg 2μg 4μg 8μg
SEL-10WDmyc
kDa
61
50
38
B
2μg
N1ICHAHis
Mock
0μg 2μg 4μg 8μg 16μg
SEL-10WDmyc
kDa
118
85
61
C
N4(int-3)CHAHis
+LacZ
N4(int-3)CHAHis
+SEL-10WDmyc
Chase(hrs) 0 0.5 1 1.5 2 2.5 0 0.5 1 1.5 2 2.5

Figure 6

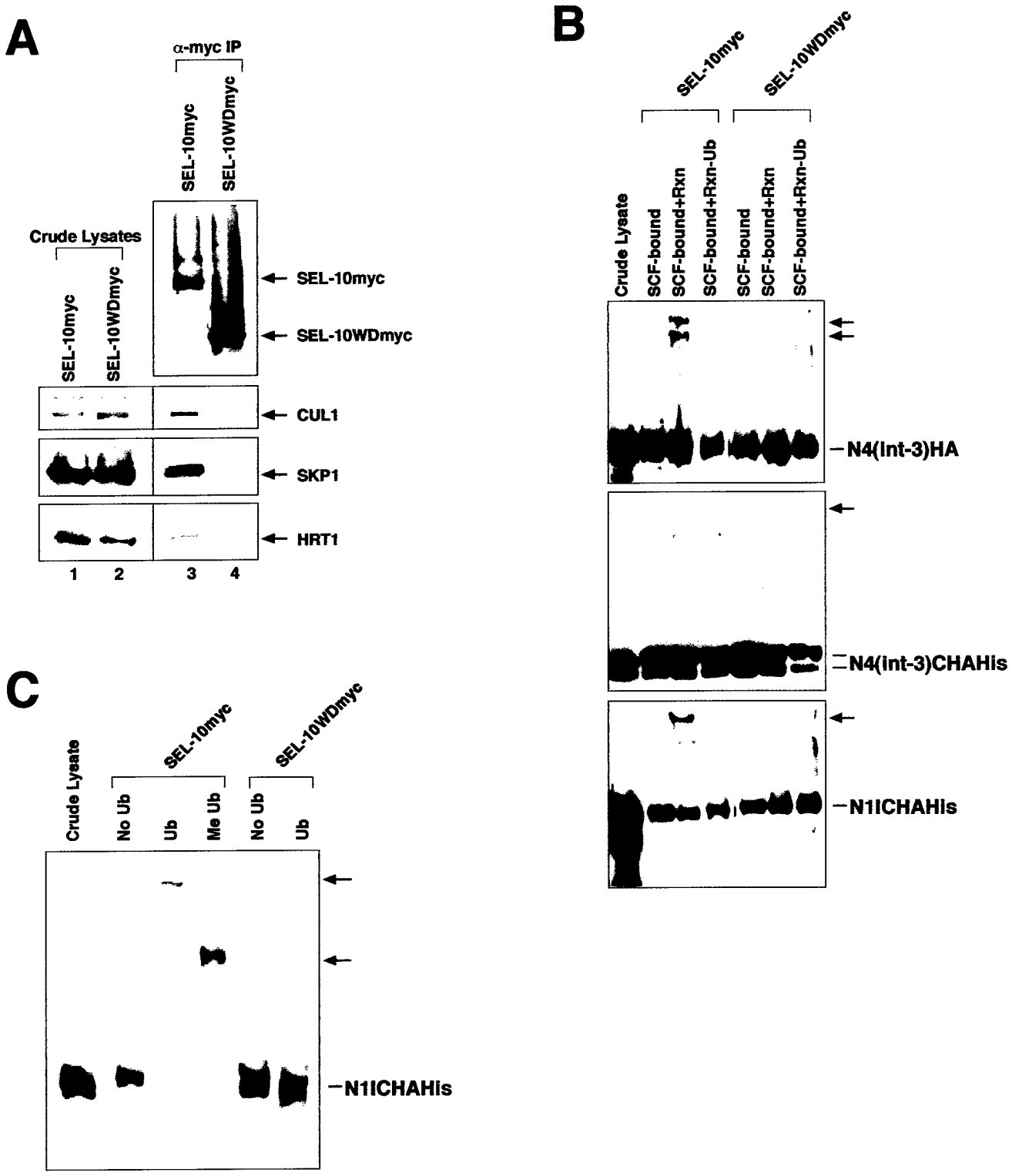

A
α-myc IP
SEL-10myc
SEL-10WDmyc
Crude Lysates
SEL-10myc
SEL-10WDmyc
SEL-10myc
SEL-10WDmyc
CUL1
SKP1
HRT1
1 2 3 4
B
SEL-10myc
SEL-10WDmyc
Crude Lysate
SCF-bound
SCF-bound+Rxn
SCF-bound+Rxn-Ub
SCF-bound
SCF-bound+Rxn
SCF-bound+Rxn-Ub
N4(int-3)HA
N4(int-3)CHAHis
N1ICHAHis
C
Crude Lysate
SEL-10myc
SEL-10WDmyc
No Ub
Ub
Me Ub
No Ub
Ub
N1ICHAHis

Figure 7

Publications

1. Wu, G., Lyapina S., Das I., Li J., Guerney M., Chui I., Deshaies R., and Kitajewski J. (2000). SEL-10, a negative regulator of Notch signaling, targets Notch for ubiquitin-mediated protein degradation. In preparation.
2. Uyttendaele H, Wu G, Roux F, Weinmaster G, Kitajewski JK. (1999). Notch4 and Jagged-1 induce microvessel differentiation of rat brain endothelial cells. Microvasuclar Research, in press.
3. Wu G., Hubbard E.J., Kitajewski J.K., and Greenwald I. (1998). Evidence for functional and physical association between Caenorhabditis elegans SEL-10, a Cdc4p-related protein, and SEL-12 presenilin. Proc Natl Acad Sci USA 95, 15787-91.
4. Hubbard E.J.A., Wu G., Kitajewski J.K., and Greenwald I. (1997). *sel-10*, a negative regulator of *lin-12* activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins., Genes & Development 11, 3182-93.
5. Wu G., and Kitajewski J.K. (1999). F-box/WD40 repeat proteins bind to and target Notch and beta-catenin for ubiquitination. Wnt Meeting, July 17-19 1999, Stanford, California, U.S.A.
6. Wu G., Hubbard E.J., Uyttendaele H., Greenwald I., and Kitajewski J.K. (1997) The int-3 mammary oncoprotein interacts with sel-10, a *C. Elegans* member of the CDC4 family of proteins. Mammary Gland Biology Meeting, June 1997, NIH, Bethesda, Maryland, U.S.A.

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